Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 72 DECEMBER, 1949 No. 3

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Effect of Vitamin-E Deficiency on Amount of Gonadotrophin in the Anterior Pituitary of Rats.* (17486)

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Associated with a deficiency of vitamin E in males there are degenerative changes in the germinal epithelium and disappearance of spermatogenesis without apparent morphological alteration of the interstitial cells. The failure of vitamin-E deficient female rats to become pregnant is apparently due to a disturbance of the implantation process rather than the absence of ovulation; there is no direct proof of ovarian dysfunction.(1,2) Since normal function of the ovaries and testes is dependent upon the anterior pituitary, several authors have compared the morphology or the gonadotrophic potency of the anterior pituitary of normal rats and of rats on a diet deficient in vitamin E. In males, van Wagenen(3) found castration changes in the basophils of the gland. Her results were extended and confirmed by Koneff.(4) The anterior pituitary of female E-deficient rats likewise showed the morphological changes characteristic of spaying, according to Joel.(5) Both Koneff and Joel also described a hypertrophy and a decrease in the number of oxyphils. In conformity with these findings, Nelson(6) reported that the increase in the gonadotrophic potency of the pituitary of E-deficient males approached that of the gland of castrates. He detected no difference in the amount of gonadotrophin in the pituitaries of normal and E-deficient females. However, McQueen-Williams(7) concluded that glands from E-deficient female rats contain about twice as much gonadotrophin as those from normal rats or E-deficient rats receiving the vitamin for 2 weeks or longer. In contrast with the foregoing reports, Biddulph and Meyer(8) stated that pituitary powder from vitamin-E deficient male or female rats produced the same weight change in the ovaries of hypophysectomized rats used for assay as normal pituitary powder from a corresponding sex. However, they believed that male pituitaries from E-deficient rats contained an increased amount of luteinizing (interstitial-cell stimulating) hormone. The gonadotrophin in all these experiments was assayed in immature female rats whereas Rowlands and Singer(9) employed a different method: the production of ovulation in estrous rabbits. According to their findings, the pituitary of the E-deficient female rat contained less gonadotrophin than the normal although this may have depended upon a reduction of a gonadotrophin not detectable in the assays in rats.

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* Aided by grants from E. R. Squibb and Sons, the United States Public Health Service, and the Williams-Waterman Fund of the Research Corporation.

1. Kaunitz, H., and Slanetz, C. A., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 334.

2. Blandau, R. J., Kaunitz, H., and Slanetz, C. A., *J. Nutr.*, 1949, v38, 97.

3. Van Wagenen, G., *Anat. Rec.*, 1925, v29, 298.

With the exception of the experiments of Biddulph and Meyer, all the assays in the work cited above were performed in animals with intact pituitaries. It seemed of interest to reinvestigate the problem by performing the assays in hypophysectomized male rats so that (a) any contribution of the anterior pituitary of the assay animals to the gonadotrophic effect of the glands of donors could be excluded, and, (b), interstitial-cell stimulation could be better evaluated from effects on the anterior prostate. The female donor groups were of special interest since studies(2) of their ovarian function had already been completed.

TABLE I.
Composition of Vit. E Deficient Diet.

Basal mixture	%
Casein, crude	30
Cerelose	54
Lard, commercial	10
Salt mixture (Hawk-Oser)	4
Celluration	2

Supplements of basal mixture	mg/kilo
Thiamine chloride	2
Riboflavin	4
Pyridoxine	4
Calcium pantothenate	10
p-Amino benzoic acid	300
Choline	1000
Inositol	1000
Vit. K	4
Oleum percomorphum	200

Methods. Albino rats of a highly inbred stock were maintained on the vitamin-E deficient diet given in Table I from birth until sacrificed. The control rats received the same diet supplemented by 3 mg of synthetic dl-alpha-tocopherol acetate[†] per 100 g of the diet, permitting a daily intake of 300 μg in contrast to 30 micrograms of the vitamin afforded by the deficient diet. At average ages ranging from 315 to 469 days, corresponding groups of vitamin-E deficient and control rats were sacrificed, and the anterior pituitary gland from each rat was removed, carefully dissected and weighed. The pituitaries from each group of rats were triturated together in a mortar into a homogenous fine suspension in distilled water, transferred to an ampoule, quickly frozen and dried in a frozen state under high vacuum.

For the bioassay of its gonadotrophic po-

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4. Koneff, A. A., *Anat. Rec.*, 1939, v74, 383.
5. Joel, C. A., *Monatschr. Geburtsh. Gynak.*, 1943, v116, 288.
6. Nelson, W. O., *Anat. Rec.*, 1931, v56, 241.
7. McQueen-Williams, M., *Anat. Rec.*, 1934, v58 (Suppl.), 77.
8. Biddulph, C., and Meyer, R. K., *Amer. J. Physiol.*, 1941, v132, 259.
9. Rowlands, I. W., and Singer, E., *J. Physiol.*, 1936, v86, 323.

[†] Synthetic dl-alpha-tocopherol acetate was kindly supplied by Dr. Leo Pirk of Hoffmann-La Roche.

TABLE II.
Gonadotrophic Potency of the Anterior Pituitary of Normal and Vitamin-E Deficient Rats.

		Donor rats			Wt of ant. pit.	
Group	Sex	Avg age, days	No.	Body wt,* g	Fresh,* mg/100 g	Dry, mg/100 g
Normal	M	325	12	375 ± 13.4	2.29 ± 0.11	.41
E-deficient	M	315	13	255 ± 12.3	2.86 ± 0.24	.54
Normal	F	388	12	254 ± 8.7	5.11 ± 0.04	.79
E-deficient	F	469	12	237 ± 11.5	4.35 ± 0.20	.71

Hypophysectomized male recipient rats†

	Ant. pit. powder							
No.	Source	Dose, mg	Wt of testes,* mg	P‡	Wt of sem. ves.,* mg	P‡	Wt of ant. prost.,* mg	P‡
23			121.3 ± 3.40		4.68 ± .33		10.91 ± .51	
27	Normal ♂	.1	154.2 ± 3.39	<.01	5.90 ± .36	>.6	17.07 ± .71	.1
29	E-defic. ♂	.1	170.1 ± 1.28		6.14 ± .34		19.05 ± .90	
29	Normal ♀	.2	147.2 ± 3.71	<.02	4.99 ± .23	>.5	16.88 ± .76	>.2
29	E-defic. ♀	.2	162.6 ± 5.10		5.19 ± .25		17.92 ± .73	

* Mean and standard error of the mean.
† Average wt range of experimental groups: 40.2 to 41.0 g; average wt of controls: 35.9 g.
‡ Comparison of means calculated according to "t" method of Fisher.

tency, a fine suspension of the dried powder in physiological saline was made so that each 2 cc contained the total amount of the gland to be injected into one rat. The total dose for groups of rats for each day was placed in separate vials which were kept frozen until injected. One-half cc of the suspension was given subcutaneously on each of the 4 consecutive days, starting one day after operation, to a male weanling rat hypophysectomized at 21 days of age by the parapharyngeal approach.(10) The rats were sacrificed 24 hours after the last injection; the testes, seminal vesicles and anterior prostates were carefully removed, freed of connective tissue and weighed on a sensitive torsion balance. Any rats with remnants of the pituitary as detected by examination of the sella with a binocular microscope were excluded. A considerable number of hypophysectomized rats was sacrificed prior to the experiments to determine a suitable dose of pituitary powder necessary to initiate hypertrophy of the sex glands. It was found to be 100 μg of the male and 200 μg of the female dried pituitary powder.

Results. It will be seen from Table II that the weight of the testes of the immature hypophysectomized rats which received injections of anterior pituitary glands from either male or female vitamin-E deficient rats was greater than that of rats receiving corresponding doses of the gland from normal male or female rats. The weights of the seminal vesicles and anterior postrates of the rats which received anterior pituitary from normal or vitamin-E deficient male and female donors showed, however, no significant difference.

Discussion. It is evident from the foregoing results that there was a significant increase in the gonadotrophic potency of the anterior pituitary glands of vitamin-E deficient rats of both sexes. These findings, however, did not indicate whether the increased gonadotrophin content of the anterior pituitary of the vitamin-E deficient rats was owing to an increased production or to a decreased secretion or to both.

The fact that only the weights of the testes and not those of the anterior prostates or seminal vesicles of the hypophysectomized rats were greater in rats which received injections of anterior pituitary from vitamin E-deficient

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10. Smith, P. E., *Am. J. Anat.*, 1930, v45, 205.

rats might be attributed to an increase in the pituitary follicular stimulating hormone (FSH) without concomitant increase in the interstitial-cell stimulating hormone (ICSH). Such an interpretation would be in conformity with the well known fact that vitamin-E deficiency causes degenerative changes of the germinal epithelium without evident histological or functional changes in the interstitial cells in the testes. It is possible that differences in the quantities of the two gonadotrophins could be revealed if several different dose levels were to be used for the comparisons. However, no conclusion can be drawn from the experiments reported in Table II.

That the anterior pituitary of the vitamin-E deficient female rats also contained an increased amount of gonadotrophic hormone is of interest because of the controversy concerning ovarian function in vitamin-E deficiency. Although the ovaries of old, E-deficient rats showed a greater accumulation of connective tissue and pigmentation than those of controls, observations of ovarian function, such as the production of ova followed by fertilization, revealed no abnormalities. It remains to be investigated, however, whether the increased gonadotrophin content of the pituitary gland indicates a disturbance of ovarian function or whether a different explanation must be sought.

Summary. The gonadotrophin content of the anterior pituitary taken from vitamin-E deficient rats, 315 to 469 days of age, was determined. The weights of testes, seminal vesicles and anterior prostates of groups of male rats hypophysectomized at 21 days of age and subsequently injected with suspensions of frozen-dried anterior pituitaries were compared. The testes of the hypophysectomized rats which received the pituitary suspension from vitamin-E deficient male or female rats were significantly heavier than those of rats which received pituitary powder from the controls. There was no difference in the weights of the seminal vesicles or anterior prostates.

It is concluded that the gonadotrophin content of the anterior pituitary gland is elevated in older vitamin-E deficient male and female rats. The relationship of these findings to the changes in the gonads in vitamin-E deficiency are discussed.

Received Nov. 15, 1949. P.S.E.B.M., 1949, v72.

A Probable Acid Mucopolysaccharide Present in Granulation Tissue.*

(17487)

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(Introduced by M. Heidelberger)

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This paper is a preliminary report of observations made on the healing of experimental fractures in the rabbit. Certain histological and chemical reactions of the reparative tissue have led us to the belief that there is present in the early granulation tissue of bone repair, an acid mucopolysaccharide which may play a role in tissue healing. Tissue sections of rabbit callus from one to 10 days after fracture were stained metachromatically with a 1% solution of toluidine blue. Early specimens take up only a small quantity of the dye. As the granulation tissue forms, however, an increasing metachromasia appears, and as the fibrous connective tissue becomes more abundant and differentiated, the ability to stain metachromatically with toluidine blue diminishes. Lison(1) attributed this ability to stain metachromatically to the presence of sulphate radicals. Wislocki, *et al.*(2) have shown, however, that hyaluronic acid, a mucopolysaccharide which contains no sulphate radical, also stains with toluidine blue. This gradual change in staining characteristics toward toluidine blue probably indicates a change in the nature of the ground substance.

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* This work was made possible by a grant through the Office of Naval Research, United States Navy Department.

1. Lison, L., *Soc. de Biol. Compt. Rend.*, 1935, v118.

Micro sections of the rabbit callus from the first to the sixteenth day after fracture were stained with the Hale technic. Hale(3) feels that this stain is specific for acid mucopolysaccharides. An acid polysaccharide such as hyaluronic acid combines with dialyzed iron in an acid solution but a neutral polysaccharide or a protein does not. The bound iron can be demonstrated as Prussian Blue by treatment with potassium ferricyanide and hydrochloric acid. If the slides are counterstained with a red dye such as paracarmine, the acid polysaccharides appear blue against the red background of other tissue structures. The positive reaction for acid polysaccharides parallels those obtained with toluidine blue. Recently injured tissue had little affinity for the dialyzed iron, but by 7 days the formed granulation tissue stained a deep blue strongly suggesting the presence of a high concentration of acid polysaccharides. Staining was most intense at 7 to 10 days, declined thereafter, and was almost disappearing by the tenth to fourteenth day.

Qualitative studies of the 7 day rabbit callus showed a high concentration of sulphate and phosphate ions. A test for the presence of amino sugars with Ehrlich's reagent was strongly positive. Believing that possibly we were staining one of the known acid mucopolysaccharides we attempted chemical isolation. A chemical analysis for hyaluronic acid by viscosimetric methods gave a negative result. Since great loss is entailed in these methods, we incubated tissue sections of the 7 to 10 day rabbit callus with increasing concentrations of hyaluronidase for varying periods of time. The tissue's staining did not change. Viscosimetric analysis by means of an Ostwald viscosimeter showed no depolymerization of the ground rabbit callus by hyaluronidase.

Following Meyer's procedure(4) attempts were made to isolate chondroitin sulphuric acid from the cartilagenous 7 day rabbit callus. The results were negative although this mucopolysaccharide might logically be present since it is seen so abundantly in mature cartilage. Repeated attempts at identification for chondroitin sulfuric acid gave negative results. A possible explanation may be that procedures for the isolation of mucopolysaccharides entail great loss and as yet, we have not had available, the necessary large quantities of tissue.

In the course of analysis for chondroitin sulfuric acid a granular substance precipitated out of slightly alkaline alcohol. This substance prolonged the Lee White clotting time of blood almost fifteen times. The addition of both protamine and toluidine blue would inhibit this action. An analysis for heparin following Howell's(5) original method yielded a small quantity of protein contaminated crystals which possessed mild anticoagulant powers.

The 7 day rabbit callus was also incubated with lysozyme. Histochemically and viscosimetrically no depolymerization of the ground substance took place. These studies indicate the probable absence of hyaluronic acid and the substrate of lysozyme. The enzyme lysozyme is present in the granulation tissue of the rabbit callus, however, in a concentration of about 84 units after the fifth day after fracture.(6) The presence of chondroitin sulfuric acid is still unsettled, but much larger quantities of tissue must be studied.

We have studied the healing cycle in the rabbit and have sought to identify by histochemical and chemical methods an acid mucopolysaccharide which appears as an intermediary metabolite in the transformation of the interfibrillar ground substance. The studies are incomplete but contribute to the clarifica-

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2. Wislocki, G., Bunting, G. H., and Dempsey, E. W., *Am. J. Anat.*, 1947, v81, 1.

3. Hale, C. W., *Nature*, London, 1946, v157, 802.

4. Meyer, Karl, and Smyth, Elizabeth M., *J. Biol. Chem.*, 1937, v119, 507.

5. Howell, W. H., *Am. J. Phys. Proc.*, 1922-1923, v63, 434.

6. Prudden, J. F., Lane, N., and Meyer, Karl, in press.

tion of the changes during healing in the organic matrix. To integrate these observations with present knowledge, a working hypothesis must take into account enzyme action, hydrogen ion concentration, and the concentrations of ions such as calcium, carbonate, and phosphate as well as other electrolytes participating in the transformation of the organic matrix. Further studies are indicated and are in progress. Because of the difficulties involved in obtaining large quantities of healing callus, the identification of the acid mucopolysaccharide is incomplete.

Summary. Chemical and histochemical observations concentrate on a mucopolysaccharide which appears to be important in bone healing.

Received July 25, 1949. P.S.E.B.M., 1949, v72.

Effect of Dietary Restriction on Susceptibility of Mice to Infection with Theiler's GDVII Virus.* (17488)

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That nutritional deficiencies are frequently accompanied by anorexia is well known. Numerous reports(1-7) have been published which concern the relation of nutritional inadequacies both to bacterial and to viral infections, but the evidence as to whether or not the course of experimental infections is influenced by caloric restriction, or by restriction of an adequate diet, inanition, seems equivocal. Different infective agents, different host species, genetic status of host and of pathogen, different routes of infection, and many other important variables frequently make direct comparison impossible and analysis of conflicting results exceedingly difficult. The experiments here reported, query further the relative effects of reduced food intake resulting from loss of appetite and of a simple decrease in caloric intake on the course of Theiler's GDVII encephalomyelitis in mice.

Materials and methods. Mice: Webster Swiss mice, from our own colony, were used in all experiments. When 3 to 4 weeks old, they were removed from the colony, weighed and distributed in individual screen-bottom cages. Each group contained approximately equal numbers of male and female mice of similar weight and litter mates in any one group were kept to a minimum.

Diets: the diet referred to as "optimum" has the following percentage composition; casein 18† sucrose 72.2, salts IV 4,(8) vitamins 0.5,‡ choline chloride 0.3 and corn oil 5.§ The vitamins were made up with sucrose so

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* Aided by a grant from the National Foundation for Infantile Paralysis.

1. Aycock, W. L., and Lutman, G. E., *Am. J. Med. Sci.*, 1944, v208, 389.

2. Clark, P. F., McClung, L. S., Pinkerton, H., Price, W. H., and Schneider, H. A., *Bact. Rev.*, 1949, v13, 99.

3. Rasmussen, A. F., Jr., Waisman, H. A., Elvehjem, C. A., and Clark, P. F., *J. Inf. Dis.*, 1944, v74, 41.

4. Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *J. Exp. Med.*, 1944, v79, 221.

5. Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *J. Exp. Med.*, 1944, v80, 257.

6. Weaver, H. M., *Am. J. Dis. Child.*, 1945, v69, 26.

7. Metcoff, J., Darling, D., Wilson, D., Lapi, A., and Stare, F. J., *J. Lab. Clin. Med.*, 1949, v34, 335.

† A hot alcohol-extracted, vitamin test casein (General Biochemicals, Inc., Chagrin Falls, Ohio).

‡ We are indebted to Merck & Co., Rahway, N.J., for the crystalline vitamins.

8. Lichstein, H. C., McCall, K. B., Kearney, E. B., Elvehjem, C. A., and Clark, P. F., PROC. SOC. EXP. BIOL. AND MED., 1946, v62, 279. In these studies the salt mixture was modified by using $ZnSO_4$ insted of Zn Cl_2.

TABLE I.
Dietary Regimen.

Ration No.	Type of restriction	Ration fed per mouse per day
1	Caloric	1 g FPSV + 0.5 g sucrose
2	”	1 g FPSV + 1.5 g sucrose
3	Total	1 g optimum ration
4	”	2 g optimum ration
5	”	3 g optimum ration
6	None	Optimum ration *ad lib.*

that 0.5 g of the mixture contained the following: thiamine 0.3 mg, riboflavin 0.3 mg, pyridoxine 0.3 mg, niacin 1.5 mg, calcium pantothenate 2.0 mg, i-inositol 100 mg, sodium p-amino-benzoate 100 mg, biotin 10 γ, folic acid 25 γ. In the rations designed to determine the effect of caloric restriction, fat, protein, salts, and vitamins (referred to as FPSV) were fed in the same proportions as they occur in the optimum diet, and the indicated amounts of sucrose were added. Each gram of the FPSV mixture contained casein 0.65 g, salts IV 0.14 g, vitamins 0.029 g and corn oil 0.18 g, enough to reasonably insure the consumption of as much of each per mouse as would be consumed by mice receiving the optimum diet *ad lib.* This being the case, the only constituent deficient in rations 1 and 2 was sucrose. The dietary regimen of each group is listed in Table I. In previous experiments of a similar nature it was learned that mice would die if placed abruptly on the restricted rations, therefore a plan of progressive restriction as outlined in Table II was followed during the first ten days of the experiment. For caloric restriction the FPSV mixture and sucrose were measured separately and blended manually in each ration cup. Groups 1 through 4 received the same quantity of FPSV. For total restriction, decreasing amounts of the optimum diet were fed until the determined level of food consumption was reached. Water was always available. Five days after the start of the experiment, the individual weights of the majority of the animals had reached a maximum (except the optimum *ad lib.* group) and by the seventh day, they had begun to decline.

Virus: 16 days after beginning the experiment all the test groups were inoculated intracerebrally with 0.03 ml of a saline suspension (Series 98, 10^{-2}; Series 115, 10^{-3}) of Theiler's(9) GDVII virus infected brain and cord. All animals were observed daily and weighed twice weekly.

Results and discussion. The results are summarized in Table III. Uninoculated mice remained alive throughout the experiment and were alert and active in contrast to those receiving the virus. In the latter group, hindleg weakness, tremors, ataxia, humped back, and occasionally tonic convulsions, with extension of the hindlegs and flexion of the forelimbs, were observed. During the convulsions, the mice became rigid and cyanotic; death sometimes occurred during these seizures, but more often the mouse recovered. Similar convulsions have been seen in other nutritional deficiencies such as that of tryptophan. In the present experiments, the animals that were paralyzed and those that showed only signs of encephalitis were grouped together in calculating the percentage virus infection.

In *Series 98,* in which practically 100% of the mice in the control inoculated groups developed signs of the virus infection, Groups 2 and 6 showed this typical infection in only 50% and 43%, respectively, but showed total fatalities in 100% and 95% of the animals. This would seem to indicate that either a diet with low caloric value or a restriction of the optimum diet to 1 g per day definitely alters the signs of infection. But, in these same groups, animals dying without showing any signs of infection survived 5 and 7.5 days (average survival time, AST) respectively while the average incubation period (AIP) of mice which did show the typical picture was 9 and 8.8 days respectively. We assume that in most cases the mice which died without signs of infection actually succumbed to virus invasion. Several lines of evidence support this assumption. Gard(10) has presented evi-

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§ Mazola, fortified with oleum percomorphum (Mead Johnson & Co., Evansville, Ind.) to supply *ca.* 1800 I.U. vitamin A and 260 I.U. vitamin D per 100 g ration.

9. Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, v72, 49.

10. Gard, S., *J. Exp. Med.*, 1940, v72, 69.

TABLE II.
Plan of Restriction.

Group No.	Days after start of experiment					
	0	3	4	5	7	10
1 and 2	Opt. *ad lib.*	Opt. 3 g	FPSV 1 g + sucrose 2 g	FPSV 1 g+ sucrose 1.5 g	FPSV 1 g + sucrose 1 g	FPSV 1 g + sucrose 0.5 g
3 and 4	,,	,,	,,	,,	FPSV 1 g + sucrose 1.5 g	FPSV 1 g + sucrose 1.5 g
5 and 6	,,	,,	Opt. 2.5 g	Opt. 2 g	Opt. 1.5 g	Opt. 1 g
7 and 8	,,	,,	,,	,,	Opt. 2 g	Opt. 2 g
9	,,	,,	Opt. 3 g	Opt. 3 g	Opt. 3 g	Opt. 3 g
10	,,	Opt. *ad lib.*	Opt. *ad lib.*	Opt. *ad lib.*	Opt. *ad lib.*	Opt. *ad lib.*

TABLE III.
Summary of Fatalities and Virus Infection.

Series No.	Group No.[*]	Ration No.	No. of mice	Fatalities,[†] %	Virus infection, %	Total fatalities, %	AST,[‡] days	AIP,[§] days
98	1 U	1	7	0	—	0	—	—
	2 I	1	20	50	50	100	5.0	9.0
	3 U	2	7	0	—	0	—	—
	4 I	2	21	0	100	100	—	7.7
	5 U	3	7	0	—	0	—	—
	6 I	3	21	52	43	95	7.5	8.8
	7 U	4	7	0	—	0	—	—
	8 I	4	20	0	100	100	—	7.9
	9 I	5	14	7	93	100	12.0[‖]	8.9
	10 I	6	14	0	100	100	—	8.9
			138					
115	1 U	1	7	0	—	0	—	—
	2 I	1	21	14	86	100	10.0	9.5
	3 U	2	7	0	—	0	—	—
	4 I	2	21	5	95	100	7.0[‖]	9.1
	5 U	3	6	0	—	0	—	—
	6 I	3	20	35	65	100	9.0	10.2
	7 U	4	7	0	—	0	—	—
	8 I	4	20	5	95	100	10.0[‖]	9.9
	9 I	5	14	7	93	100	7.0[‖]	10.0
	10 I	6	14	14	86	100	8.0	11.0
			137					

[*] U = uninoculated; I = inoculated.
[†] Without signs of infection.
[‡] Average survival time of mice dying without signs of GDVII infection.
[§] Average incubation period of mice developing signs of GDVII infection.
[‖] 1 mouse only.

dence that the AIP is an index of virus titer. On numerous occasions we have assayed the brains and cords of infected, undernourished mice at intervals after inoculation and when moribund but without typical signs of infection. This central nervous system material when subsequently inoculated into normal mice has shown virus activity indicating that the virus does proliferate in the CNS of deficient mice even though the typical signs of infection are not seen. Further, in some experiments, deficient inoculated mice which have survived beyond the normal incubation period without visible evidence of infection have succumbed to the disease with the typical picture when fed optimum rations. As criteria of infection, therefore, we are inclined to weigh not only paralysis and visible signs of encephalitis, but also the AIP, the AST and total fatalities.

For example, in this series 98, although groups 2 and 6 showed only 50 and 43% respectively of manifest virus infection, the AIP, the AST, and the total fatalities present a picture of almost 100% actual virus infection. The results obtained with Groups 9 and 10 are essentially alike with almost 100% manifest virus infection. A number of mice receiving 3 g of optimum diet per day did not consume this amount from one feeding to the next; it is inferred therefore that their appetites were satisfied, approximating the *ad lib*. group in food consumption. The groups receiving 1 g or 2 g of optimum diet per day devoured their ration completely. The remaining groups(4,8) with less severe restrictions, showed no significant differences in incidence of paralysis.

Series 115. Thirty-five % of the mice in group 6 (1 g optimum diet) died without any signs of the disease. However, the AST (9.0 days of these mice was less than the AIP (10.2 days) for this group and both the AST and AIP were less than the AIP (11.0 days) of the animals on the optimum ad lib. diet (Group 10). Among the remaining groups (2, 4, 8) in this series, typical infection levels ranged from 86% to 95%.

We have therefore almost identical pictures in the two series. In each, the groups receiving restricted amounts of food, whether low in calories, or low in amounts of an optimum diet, showed no significant decrease in infection as we have defined the term. The exact figures for the two series are not directly comparable since in series 98, the inoculum was a 10^{-2} suspension whereas in series 115, this was a 10^{-3} suspension. This difference resulted in a longer AIP and a slightly longer AST in the latter series. Our results are not precisely in accord with the reports of others who have found that restriction of the caloric value or reduction of food intake produces results similar to but less marked than those obtained with thiamine deficient diets. We would emphasize that our criteria of infection do not rest solely on visible signs and that the other authors used different viruses, either Theiler's FA or the Lansing mouse adapted poliomyelitis virus. Only with the Lansing strain did they obtain definitive results.

Although Theiler(11) has reported that the more obvious physical and chemical properties of mouse encephalomyelitis virus and human poliomyelitis are "identical", immunologically, and on the basis of species susceptibility and the pathological lesions produced, the viruses are definitely different. The dissimilarity in behavior of GDVII and Lansing poliomyelitis virus in mice when the animals are under various nutritional stresses appears to be another manifestation of differences between these viruses. Our experience indicates that the course of infection with the Lansing strain is more sensitive to nutritional stress than is infection with Theiler's GDVII virus.

Summary. Although diets restricted either calorically or by total food intake do influence the course of infection in mice inoculated with Theiler's GDVII virus, in that frank signs of infection, *i.e.* paralysis, encephalitis, are frequently not evident, yet the total fatalities, the average incubation period, and the average survival time are not modified appreciably by these deficiencies.

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11. Theiler, M., *Med.*, 1941, v20, 443.

Received August 3, 1949. P.S.E.B.M., 1949, v72.

Monkey Pathogenicity of Various Strains of Murine Poliomyelitis Virus. I. Experiments with Lansing, MEF, and Yale-SK Group.* (17489)

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Adaptation of human poliomyelitis virus from monkey or man to rodents results in the production of murine strains which differ significantly in the degree of their pathogenicity for mice and rhesus monkeys. One group, with low potency in mice, *i.e.* Lansing, MEF and Yale-SK virus, retain to a large extent their original ability to paralyze rhesus monkeys, even though continuous passage from mouse to mouse may cause some deterioration of this property.(1) Another group, Columbia-SK and MM virus, with high potency in mice, produce in rhesus monkeys rarely flaccid paralysis but set up a subclinical infection characterized by massive intraneural and extraneural virus multiplication as well as by abundant antibody formation.(2) A third group, transferable only to suckling mice, apparently has no pathogenicity whatsoever for rhesus monkeys.(3) The following communication deals with a study of the pathogenicity for rhesus monkeys of serial mouse passages of viral strains belonging to the first group, *i.e.* Lansing, MEF and Y-SK virus.

Experiments with the Lansing Virus. The strain was obtained in 1945 in its 36th mouse passage through the courtesy of Dr. G. Dalldorf, State Laboratory, Albany, N. Y. The virus has since been carried in this laboratory in mice by making transfers at irregular intervals from glycerinated material stored in the ice-box. A 10% suspension of infected brain and cord tissues was used routinely and 0.03 cc injected intracerebrally into 11-14 g Swiss albino mice. During the past 3 years the virus was examined for its pathogenicity for rhesus monkeys on 3 occasions. In each case a 10% suspension was prepared from freshly harvested brain and cord of a paralyzed mouse and 1 cc of the supernatant, after light centrifugation, was injected intracerebrally into rhesus monkeys weighing about 2500-3000 g. One monkey was injected with the 41st, 2 monkeys with the 57th, and one monkey with the 63rd serial mouse passage. None of the injected monkeys showed any signs of disease, such as fever, weakness or paralysis. Since the animals were subsequently used for the production of hyperimmune anti-Lansing serum there was no opportunity for examining any sections of the spinal cord for possible subclinical lesions or for testing these animals for immunity by reinfection with potent simian virus.

In subsequent experiments the same strain was used in attempts to infect 2 cynomolgus monkeys by intracerebral injection with 1 cc of a 10% mouse brain suspension from the 74th serial mouse passage. Both monkeys remained free from symptoms. It will be of considerable interest to further examine this apparently monkey-nonpathogenic Lansing strain for its ability to induce protection, in the absence of paralysis, against a pathogenic strain.

At the time these observations were made the virus always presented the typical clinical, biological and serological characteristics of the strain. Paralysis occurred only after intracerebral and not intraperitoneal injection, infected mice showed flaccid paralysis of the front or hind legs, the incubation period varied from 48 hours to 21 days, and the potency of the virus fluctuated between 10^{-1} and 10^{-2} with a mortality rate of between 80 and 90%. Moreover, the virus was neutralized by hyperimmune Lansing antiserum (obtained from Dr. Isabel Morgan, Johns Hopkins University, Baltimore) and by a majority of normal and polio-convalescent sera collected in the metropolitan area, but not by Col-SK antiserum or

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* Aided by a grant from the Sister Elizabeth Kenny Foundation.

1. Theiler, M., *Medicine,* 1941, v20, 43.

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3. Dalldorf, G., Sickles, G. M., Plager, H., and Gifford, R., *J. Exp. Med.,* 1949, v89, 567.

by GDVII antiserum.†

Experiments with the MEF Virus. The strain was obtained in 1945 in its 8th mouse passage through the courtesy of Dr. P. K. Olitsky, Rockefeller Institute, New York City. The virus was carried in mice in this laboratory using the same technic as previously described for the Lansing virus, except that passages were made rapidly with freshly harvested material and no intervening storage. On 4 occasions the virus was examined for pathogenicity in rhesus monkeys by intracerebral injection of 1 cc of 10% viral mouse brain-cord suspension. One monkey received the 10th, one monkey the 15th, one monkey the 19th, and one monkey the 20th serial mouse passage. The first monkey injected with the 10th serial mouse passage became paralyzed on the 14th day but no active virus could be recovered from the cord of the paralyzed animal by transfer to another rhesus monkey or to a group of mice. The other three monkeys remained free from any symptoms of disease. Two of the symptomless monkeys were tested, two months later, for immunity by intracerebral challenge with Aycock virus (0.5 cc 1:100). Both developed typical paralysis. The third monkey was used for the production of hyperimmune anti-MEF serum.

As previously described for the Lansing virus, the MEF virus at all times maintained its typical clinical, biological and serological characteristics.‡

Experiments with the Y-SK Virus. The virus was obtained in 1947 through the courtesy of Dr. G. Dalldorf. It was propagated in this laboratory by rapid serial passage in mice as previously described for the MEF virus. Intracerebral tests for pathogenicity in rhesus monkeys were carried out with various generations, inoculating two monkeys with the 5th and one monkey each with the 10th and 15th serial mouse passages. All monkeys developed typical flaccid paralysis within 9 to 13 days after injection, except one monkey inoculated with the 5th passage and another monkey inoculated with the 10th passage. No attempts were made to carry the strain further from monkey to monkey but all efforts to recover the virus by inoculation of mice with the cord of paralyzed monkeys uniformly failed.

The Y-SK virus was obtained once more in 1948 through the courtesy of Dr. J. Melnick, Yale University, New Haven, Conn., the material being received in form of frozen pooled mouse brains and cords. The original material proved highly infectious for rhesus monkeys, since 7 of 8 monkeys injected intracerebrally on various occasions between 1948 and 1949 developed typical paralysis. The strain was carried in mice in this laboratory by rapid transfer from mouse to mouse using freshly harvested brains and cords. Tests for rhesus pathogenicity were made with the 6th, 11th and 15th serial mouse passage. One of 2 monkeys each injected with the 6th or 11th mouse passage developed paralysis, as did 2 of 3 monkeys injected with the 15th mouse passage. Again, virus in mouse-pathogenic form could not be recovered from the cords of these animals when completely paralyzed.

During the time of these observations the Y-SK virus maintained its typical clinical, biological and serological characteristics, which made it indistinguishable from the Lansing and MEF virus, except for its somewhat lower virulence in mice.

Conclusions. The data presented above demonstrate that strains of mouse-adapted human poliomyelitis virus may exhibit wide variations in the degree of their pathogenicity for rhesus monkeys. The Lansing virus, for instance, harvested from remote mouse passages, gave no evidence of being pathogenic for rhesus monkeys in repeated tests. The MEF virus, originally rhesus-pathogenic, apparently suffered a progressive loss of its pathogenicity for rhesus monkeys during serially maintained rapid mouse passages. The Y-SK virus, finally, seemed to have undergone but little change in its pathogenic power for rhesus monkeys as the strain was passed rapidly through mice. It is realized that the described phenomena may not always be duplicated in different laboratories since the Lansing virus has frequently been reported by others as being fully rhesus-pathogenic. Yet the authenticity of our strains is attested by the fact that all three murine strains had fully preserved their biological and immunological characteristics. The observed differences in the degree of pathogenicity for rhesus monkeys and in the extent to which murine virus returns to mice from paralyzed monkeys may be due to individual differences among strains or reflect an attainment of different levels of perfection in the adaptive process.

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† This statement refers to the strain under investigation in this paper and not to any rhesus-nonpathogenic, high-titred, serologically atypical possible variants which may or may not have been derived from the original Lansing virus.(4,5,6)

4. Jungeblut, C. W., *Proceedings Fourth Internat. Congress for Microbiology*, Copenhagen, 1947, p. 259.

5. Enright, J. B., and Schultz, E. W., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 541.

6. Sanders, F. K., *Proceedings Federation of American Societies for Experimental Biology*, Atlantic City, 1948, p. 309.

‡ Reference is made to the original MEF strain and not to a rhesus-nonpathogenic, high-titred possible variant which has previously been described.(4)

Received Sept. 8, 1949. P.S.E.B.M., 1949, v72.

Monkey Pathogenicity of Various Strains of Murine Poliomyelitis Virus. II. Experiments with Col. SK-MM Group.* (17490)

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In the preceding paper(1) data have been presented to show that the Lansing, MEF and Y-SK virus, upon serial passage in mice, may undergo various degrees of deterioration in their ability to produce flaccid paralysis in intracerebrally infected rhesus monkeys. This reduction in monkey pathogenicity, however, was not associated with any demonstrable changes in other biological or immunological properties. It became next of interest to investigate whether the Col-SK-MM group of viruses—agents which since their initial transfer to mice have shown little or no paralyzing power in rhesus monkeys—would be capable of inducing paralytic infection, with central nervous system lesions, in cynomolgus (Java) monkeys.

The cynomologus monkey was chosen for this purpose since the response of this animal to poliomyelitis virus differs in many respects from that observed in rhesus monkeys. Whereas the rhesus monkey, as a rule, is susceptible only to intracerebral infection and the virus remains strictly localized in the central nervous system, the cynomolgus monkey may be infected by peripheral portals of entry, including the gastrointestinal tract, with the production of a systemic disease characterized by wide extraneural distribution of the virus and by fecal excretion. Moreover, isolation of poliomyelitis virus from fresh human or nonhuman sources often succeeds better with the cynomolgus than with the rhesus monkey.(2)

The following communication deals with a study of the pathogenicity for cynomolgus monkeys of current mouse passages of Col-SK and MM virus. Included are also experiments with 2 viruses of as yet uncertain identity but with close serological relationship to Col-SK and MM virus. These are the virus of so-called encephalomyocarditis (EMC virus)(3) which was isolated from chimpanzees suffering from unknown myocardial disease, and the Schultz High Lansing strain, presumably a "high" variant of the original Lansing vi-

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* Aided by a grant from the Sister Elizabeth Kenny Foundation.

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2. Melnick, J., *Am. J. Hygiene*, 1949, v49, 8.

3. Schmidt, E. C. H., *Am. J. Path.*, 1948, v24, 97.

4. Enright, J. B., and Schultz, E. W., Proc. Soc. Exp. Biol. and Med., 1947, v66, 541.

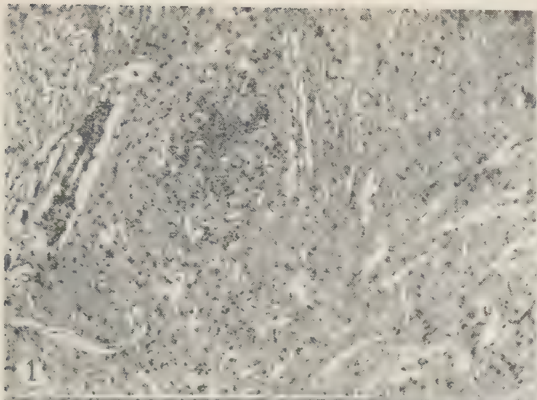
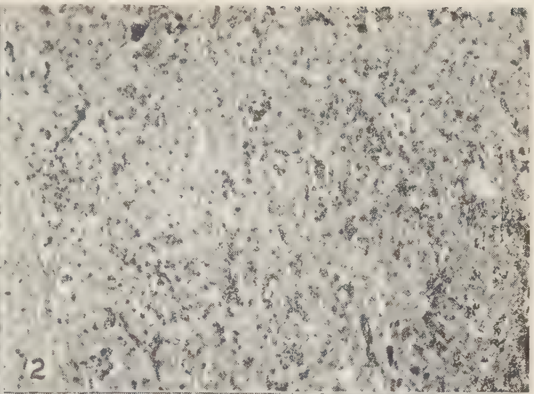
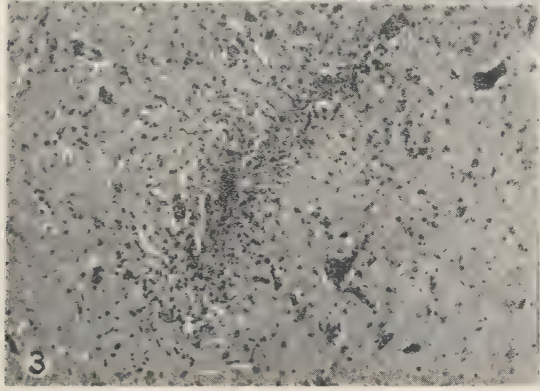
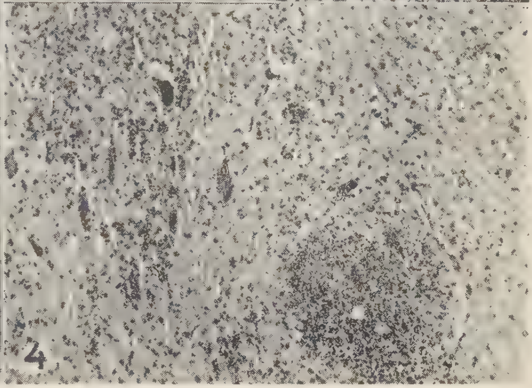





FIG. 1. Col-SK virus (guinea pig) first monkey generation cynomolgus No. 7 lumbar cord.
FIG. 2. MM virus (mouse) first monkey generation cynomolgus No. 12 lumbar cord.
FIG. 3. High Lansing virus third monkey generation cynomolgus No. 32 cervical cord.
FIG. 4. EMC virus third monkey generation cynomolgus No. 30 lumbar cord.

rus.(4) For purpose of control, 2 highly virulent Theiler strains of spontaneous mouse encephalomyelitis, *i.e.* GDVII and FA virus, were also examined for possible pathogenic effects in cynomolgus monkeys. The EMC virus was obtained from Dr. J. Warren, Army Medical School, Washington, D. C., the Schultz High Lansing virus from Dr. Edwin Schultz, Stanford University, California, and the 2 Theiler strains from Dr. Max Theiler, Rockefeller Foundation, New York.

Methods. Ten per cent viral brain suspensions were prepared from freshly harvested current mouse passages of the above mentioned strains and 1 cc of the supernatant, after light centrifugation, was injected intracerebrally into young cynomolgus monkeys weighing between 1000 and 2000 g. In the case of Col-SK virus, in addition to mouse virus preparations, viral brain suspensions were also used from paralyzed cotton rats or guinea pigs. The injected cynomolgus monkeys were kept under close observation and symptoms noted. Animals showing paralysis were sacrificed at the height of their smyptoms and the cord was removed for histological study as well as for virus recovery. Freshly prepared monkey cord suspensions were further passed to new cynomolgus or rhesus monkeys (1 cc 1:10 intracerebrally) and, at the same time, were titered out in mice by both the intracerebral and intraperitoneal route. Serial monkey passages were maintained over 2, 3 or 4 generations depending upon the absence or presence of paralytic symptoms. The results obtained are brought together in Diagram I and in Table I and are further illustrated in a series of microphotograms (Fig. 1-4).

Col-SK Virus. It will be noted that Col-SK virus, whether harvested from paralyzed cot-

TABLE I. Symptoms, Pathology, and Viral Content (Cord) of Monkeys Paralyzed by Col-SK, MM, High Lansing (Schultz) or EMC Virus.

Monkey No.	Virus injected	Monkey generation	Symptoms	Pathology, CNS	Virus content of cord titered in mice	
					i.c.	i.p.
Cynomolgus C2	Col-SK cotton rat No. 5	I	Generalized paralysis, 4 days	Ganglion cell necrosis	10^{-5}	10^{-5}
Rhesus AS48	"	I	Paresis both legs, 7 days	Died 16 days. Poliomyelitis with repair	Neg. 10^{-1}	Neg. 10^{-1}
Cynomolgus C4	Col-SK mouse No. 422	I	Paralysis left arm and both legs, 3 days	Recovered	N.d.†	N.d.†
" C5	"	I	Paralysis right leg, 3 days	Typical poliomyelitis	10^{-5}	10^{-5}
" C1	Col-SK guinea pig No. 81	I	Paralysis left arm and leg, 6 days	" "	10^{-5}	10^{-3}
" C6	" No. 83	I	Weakness 5th day, died 6th day	" "	10^{-3}	10^{-1}
" C7	" No. 83	I	Paralysis right leg, 13 days	" "	10^{-1}	Neg. 10^{-1}
Rhesus AS50	Col-SK monkey C5	II	Paralysis right leg, 6 days	" "	Neg. 10^{-1}	Neg. 10^{-1}
" AS51	"	II	Paralysis right arm, 13 days	" "	10^{-1}	Neg. 10^{-1}
Cynomolgus C21	"	II	Weakness 3rd day died 3rd day	Ganglion cell necrosis	10^{-5}	10^{-5}
" C22	"	II	Generalized paralysis, 4th day	" " "		
Rhesus AS60	" C7	II	Paralysis left arm, 7 days	Typical poliomyelitis	10^{-3}	10^{-1}
" AS62	" AS51	III	Paralysis left arm, 8 days	" "	10^{-3}	10^{-1}
Cynomolgus C11	MM mouse No. 138	I	Generalized paralysis, 3 days	Ganglion cell necrosis	Neg. 10^{-1}	Neg. 10^{-1}
" C12	" " " "	I	Paralysis both legs, 4 days	Typical poliomyelitis	10^{-3}	10^{-3}
" C19	High Lansing mouse No. 9	I	Generalized paralysis, 3 days	" "	10^{-5}	10^{-5}
" C20	High Lansing mouse No. 9	I	Generalized paralysis, 3 days	Ganglion cell necrosis	N.d.†	N.d.†
" C25	High Lansing monkeys C19-C20	II	Paralysis left leg and arm, 4 days	" " "	10^{-5}	10^{-5}
" C26	High Lansing monkeys C19-C20	II	Paralysis left leg and right arm, 6 days	Typical poliomyelitis		
" C31	High Lansing monkeys C25-C26	III	Generalized paralysis, 5 days	" " "	10^{-5}	10^{-5}
" C32	High Lansing monkeys C25-C26	III	Generalized paralysis, 4 days	" " "		
" C17	EMC mouse No. 70	I	Paralysis both legs, 3 days	" " "	10^{-5}	10^{-5}
" C18	"	I	Paresis left leg and arm, 4 days	Died 24 days. Not examined	N.d.†	N.d.†
" C23	EMC monkey C17	II	Generalized paralysis, 5 days	Typical poliomyelitis	10^{-5}	10^{-5}
" C24	"	II	Paralysis left arm and leg, 5 days	Atypical		
" C30	EMC monkeys C23-C24	III	Paralysis left arm and both legs, 4 days	Typical poliomyelitis	10^{-5}	10^{-5}

† N.d = not done.

ton rats, mice or guinea pigs, proved to be highly pathogenic for cynomolgus monkeys in paralyzing 5 of 6 such monkeys following intracerebral injection; on the other hand, paralysis occurred in only one of 2 injected rhesus monkeys. When carried from the first to the second monkey generation, the number of "takes" was greatly diminished, only one of 5 injected cynomolgus monkeys and 3 of 7 injected rhesus monkeys developing paralysis. In the third monkey generation there was a further reduction of monkey pathogenicity in that paralysis occurred in one rhesus monkey only within a group of 5 rhesus and 2 cynomolgus monkeys. No paralysis was observed in the fourth monkey generation consisting of 2 rhesus monkeys.

The lesions observed in the spinal cord of paralyzed cynomolgus or rhesus monkeys are described in Table I. In 8 out of 12 cases they were those of severe acute poliomyelitis, consisting of widespread ganglion cell necrosis in the anterior horn, with numerous examples of neuronophagia, extensive inflammatory reactions around the blood vessels and occasional areas of hemorrhage. In 3 cases the pathology was limited to more sharply defined localizations of ganglion cell necrosis in the anterior horn or central grey with poorly developed neuronophagia and but little inflammatory response. One case, finally, showed a mild poliomyelitic process with evidence of beginning repair characterized by extensive gliosis. A constant feature of the severely damaged cords was the close proximity between perivascular infiltration and ganglion cell necrosis. Pathological examination of the brain of 2 cynomolgus monkeys (not listed in Table I), which had developed paralysis following intracerebral injection with 1 cc of a 10^{-2} and 10^{-3} dilution of Col SK mouse virus, showed occasional lesions in the brain stem and a few scattered collections of cells in the motor cortex, near the tract of inoculation. A typical picture has been selected for illustration in microphotogram 1.

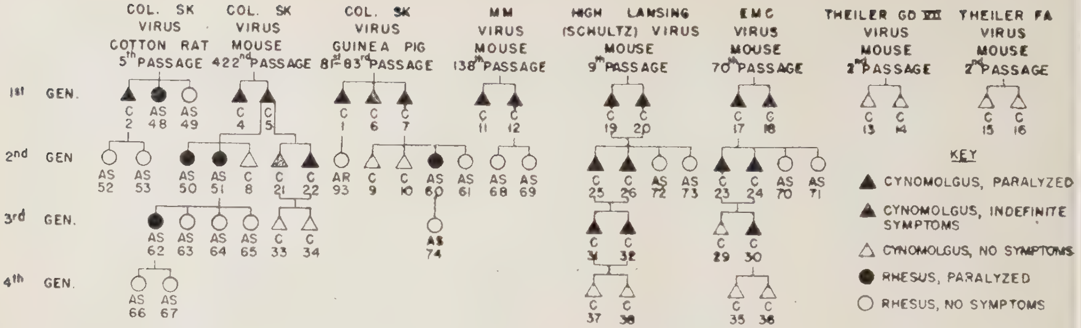
The results of virus titrations in mice injected with cord suspensions from the paralyzed monkeys are given in Table I. In general, the virus obtained from paralyzed monkeys was considerably less mouse-pathogenic than virus harvested from paralyzed mice but higher titers were usually observed in cynomolgus rather than in rhesus monkeys. With successive monkey passages virus recoveries, especially from rhesus monkeys, were of the order commonly observed with low-titred murine strains belonging to the Lansing, MEF and Y-SK group. However, when virus harvested from the original group of injected mice was further passed to a new set of mice, complete restoration of potency to the titer characteristic of Col-SK mouse passage virus, *i.e.* 10^{-9} i.c. and 10^{-8} i.p., became at once apparent. The loss of viral potency suffered by monkey passage was therefore merely a transient depression of virulence and the virus had not been transformed into a permanently low strain.

MM Virus. Two cynomolgus monkeys injected with the mouse virus became paralyzed within 3-4 days but attempts to carry the virus into the second monkey generation were unsuccessful. One of the paralyzed monkeys presented typical poliomyelitic lesions in the cord (Fig. 2), the other showed only ganglion cell necrosis with scanty inflammatory reaction. The virus recovered from the cords of both paralyzed cynomolgus monkeys proved of exceptionally low virulence in mice but could be restored to its original high level of murine potency in the next mouse passage.

High Lansing (Schultz) Virus. The mouse virus paralyzed 2 cynomolgus monkeys each in the first, second and third monkey generation but failed to transmit to the fourth generation. Severe poliomyelitic lesions (Fig. 3) were found in 4 of the 6 paralyzed monkeys, whereas in the remaining 2 animals only occasional areas showed ganglion cell necrosis without inflammatory reaction. The cords of all paralyzed monkeys yielded virus of moderately high virulence for mice.

EMC Virus. The mouse virus paralyzed one of 2 cynomolgus monkeys in the first generation, both cynomolgi of the second, and one of 2 in the third generation; no paralysis was obtained in the fourth generation. Typical poliomyelitic lesions (Fig. 4) were found in the cords of 3 animals, the remaining monkeys presenting an atypical picture of occasional chromatolysis of ganglion

DIAGRAM I



MONKEY PATHOGENICITY OF THE COL. SK—MM GROUP OF VIRUSES
FIG. 5.

cells but no perivascular infiltration. No evidence of demyelination could be detected in the cords of the 4 monkeys examined. Virus recovered from the cords of paralyzed monkeys, in all 3 generations, proved consistently moderately pathogenic for mice.

Theiler Virus (GDVII and FA). Two cynomolgus monkeys were injected intracerebrally with each of the 2 strains harvested from fresh mouse passages. None of the 4 animals showed any signs of illness during one month observation. Two monkeys were sacrificed at that time and cord sections examined histologically. No lesions of any kind were found in these cords.

Immunological Tests. The immunological properties of the various murine strains, following their passage through monkeys, were studied: 1) by examining a number of representative sera from paralyzed or symptomless monkeys for their ability to neutralize Col-SK and Y-SK murine virus in mice, 2) by determining the extent to which Col-SK monkey virus, harvested from infected monkey cords, could be inactivated in mice by known Col-SK and Y-SK antisera, 3) by reinfecting all surviving symptomless monkeys with simian poliomyelitis virus. Suffice it to say that neutralization of Col-SK murine virus was readily obtained by the majority of sera collected from cynomolgus or rhesus monkeys infected with Col-SK, MM, High Lansing or EMC virus (but not with Theiler virus), whereas the same sera failed to neutralize Y-SK murine virus. In harmony with this fact, Col-SK virus, prepared from infected cynomolgus cords, was fully inactivated by known Col-SK antiserum but not regularly by known Y-SK antiserum. In the reinfection experiment, finally, a group of 8 cynomolgus monkeys surviving asymptomatic infection with either Col-SK virus (C8, C10, C33, C34) High Lansing virus (C37, C38) or EMC virus (C29, C35), together with one normal rhesus control monkey, were challenged by intracerebral injection of simian poliomyelitis virus (1 cc 1:10 Aycock rhesus passage virus). All animals developed complete or partial paralysis within 1-3 weeks.

Discussion. The results obtained in this study leave little doubt that the murine strains at present classified as the Col-SK-MM-EMC-Mengo group of virus are highly pathogenic for cynomolgus monkeys and induce in this animal an experimental disease which is symptomatologically and pathologically similar to that produced by the Lansing-Y-SK group of murine virus in rhesus monkeys. African grey monkeys (Cercopithecus aethiops) have also been reported as developing paralysis more frequently than rhesus monkeys following infection with Mengo virus.(5) Although these facts seem well enough established,† significant differences in the degree of such monkey pathogenicity between the two

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5. Dick, G. W. A., and Smithburn, K. C., *Br. J. Exp. Path.*, 1948, v29, 547; Dick, G. W. A., *ibid.*, 1948, v29, 559.

† The fact that Col-SK virus paralyzes cynomolgus monkeys with the pathological picture of poliomyelitis in the spinal cord has been confirmed by Dr. J. D. Verlinde, Leiden, Holland, who will publish his findings elsewhere.

groups of virus must not be overlooked. For, whereas murine Lansing, MEF and Y-SK virus can often, if not always, be maintained in an unbroken series of monkey generations, murine Col-SK, MM, High Lansing and EMC virus produce paralysis in monkeys only for a limited number of passages. The abrupt extinction of paralyzing power for primates, despite the presence of a potentially virulent agent and the manifestation of extensive lesions in the central nervous system of the infected monkey, constitutes an as yet unsolved problem. The problem is further accentuated by the fact that monkey passage fails to bring about a permanent conversion of the "viscerotropic" Col-SK strain into the "neurotropic" Y-SK strain. If one contrasts the obvious depression of virulence in monkeys—a frequent phenomenon for the Col-SK-MM group of virus—with the ease with which the same viruses can be propagated in mice, one is confronted with several possible conclusions. Either different murine strains of human poliomyelitis virus possess different innate degrees of pathogenicity for monkeys and mice; or, some of these viruses have suffered an irreversible loss of their intrinsic monkey pathogenicity, subsequent to their experimental adaptation to rodents. A third consideration would be that in the case of the Col-SK-MM-EMC-Mengo group of viruses we are dealing with a serologically homogenous group of aberrant members of the poliomyelitis family which, though capable of producing paralytic poliomyelitis in monkeys and probably also in man,(6) are genetically not of human but of rodent origin in nature. This hypothesis might receive support from the recent demonstration of the regional occurrence of virucidal antibodies against EMC and MM virus in the sera of wild rats.(7) Obviously, further study of the complex problem is necessary in order to provide the correct answer.

Conclusions. 1) Col-SK, MM, High Lansing and EMC virus, harvested from remote rodent passages, produce typical poliomyelitis in intracerebrally infected cynomolgus monkeys. 2) Two strains of highly virulent Theiler virus, GDVII and FA, are non-pathogenic for cynomolgus monkeys. 3) The Col-SK-MM group of virus can be carried in cynomolgus monkeys only for a limited number of passages. 4) Judging from serological and biological characteristics, monkey passage fails to bring about a permanent conversion of Col-SK virus into Y-SK virus.

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Received September 8, 1949. P.S.E.B.M., 1949, v72.

Artificial Kidney. II. Construction and Operation of an Improved Continuous Dialyzer.* (17491)

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The insertion of a continuous dialyzer (artificial kidney) in the circulatory system of an animal or a human in uremia has been shown to be capable of removing nitrogenous waste products(1-10) and to result in the adjustment of electrolyte equilibrium.(1,3,8,

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* Supported in part by a grant from the Miles Laboratories, Elkhart, Ind.

† Biochemist, Crile Veterans Administration Hospital, Parma, Ohio.

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10,11). This continuous dialysis of blood has proved valuable(12,13) in the isolation of substances which are present in blood in low concentrations. The method may also be applied to fundamental physiological studies. (14,15) For such dialysis to be reasonably successful the apparatus used should contain a large area of dialyzing membrane so that the rate of dialysis may be rapid and the process be completed in 1-4 hours. The volume of blood which it contains should be relatively small and constant in order to avoid undue changes in the subject's blood volume. The dialyzing solution on the other side of the membrane should be in a closed system. This is necessary in order to keep a certain amount of CO_2 in solution which makes possible a physiological pH and avoids precipitation of calcium phosphate. The apparatus must be easy to clean, assemble and sterilize and should preferably be inexpensive.

Several artificial kidneys have been described.(1,2,7,12,14,16) Although differing in constructional details they are all characterized by the use of long lengths of cellophane tubing (sometimes greater than 100 feet) and the design most widely used at the present(1) is of an undesirably large size. In a previous communication(17) we described a new design of a continuous dialyzer which met the above requirements more closely than any previously described. Although it has been used in many successful experiments it had one main disadvantage in that the blood being dialyzed was exposed to a large surface of rubber which accelerated clotting and necessitated coating the rubber with a non-wetting silicone resin. This involved a considerable amount of work. The present report describes an improved model in which the above objection is overcome and in which the dialyzing efficiency has been greatly increased.

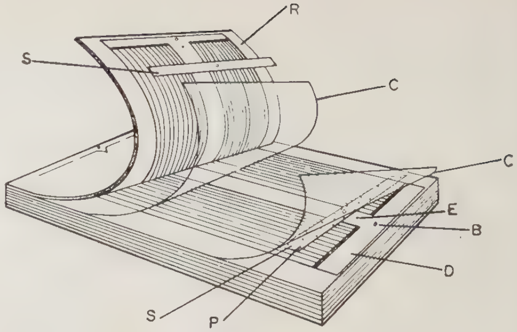

FIG. 1.

Diagram of the artificial kidney. The top rubber pad of an assembly of 4 units has been bent back to show the relative position of the inner parts.

R—Grooved rubber pads.

C—Cellophane sheets.

S—Stainless steel plates between the two sheets of cellophane. These plates are separated one millimeter by projections (P) in one of the plates.

D—Depression in rubber pads to accommodate the steel plates.

B—Interconnecting tubes for blood. The holes in the steel plates fit exactly over B.

E—Interconnecting tubes for dialyzing solution.

The dialyzer is composed of a variable number of units, each unit consisting of two identical longitudinally-grooved rubber pads‡ (12 inches by 18 inches by 3/16 inch), between which are placed 2 sheets of cellophane (Fig. 1). The two pieces of cellophane are separated at either end by 2 identical pairs of stainless steel plates which at one end provide

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‡ The authors wish to acknowledge the invaluable assistance of the Houck Machine Co., Barberton, Ohio, for machining the specially designed steel mold and of A. E. Sidnell and the personnel of the Seiberling Latex Products Co., of Akron, Ohio, for molding the rubber pads.

an opening through which blood may enter the space between the pieces of cellophane and flow in a uniform film to the opposite end where it may leave the apparatus through the second pair of steel plates. Dialyzing solution flows in a countercurrent direction through the grooved spaces in the rubber pads on both sides of the thin layer of blood contained between the two pieces of cellophane. As many such units as desired are easily assembled one above another and clamped with a simple holder composed of 2 flat steel plates. When so assembled all units are automatically connected in parallel through suitable interconnecting holes at both ends of the rubber pads. By appropriate placement of small slips of cellophane between the individual units, they may be connected in series or in series parallel, several combinations being possible. This makes it possible to alter the resistance of the "kidney" to the available flow of blood. For dogs weighing between 15 and 20 kg in which blood is drawn from the femoral artery and returned to the femoral vein the best assembly has been found to be a total of 8 units, 2 parallel sets of 4 units being connected in series.

If the "kidney" is properly assembled, there will be no leakage of blood into the dialyzing solution, or between the rubber pads. To detect assembly errors, air is introduced into the spaces to be occupied by the blood at 150-200 mm of mercury above atmospheric pressure. If no decrease in air pressure occurs, the apparatus is considered satisfactory. No holes have yet been found in the cellophane sheets and leaks have never developed during the dialysis. DuPont No. 300 non-moisture-proof cellophane sheets may be purchased cut to size.

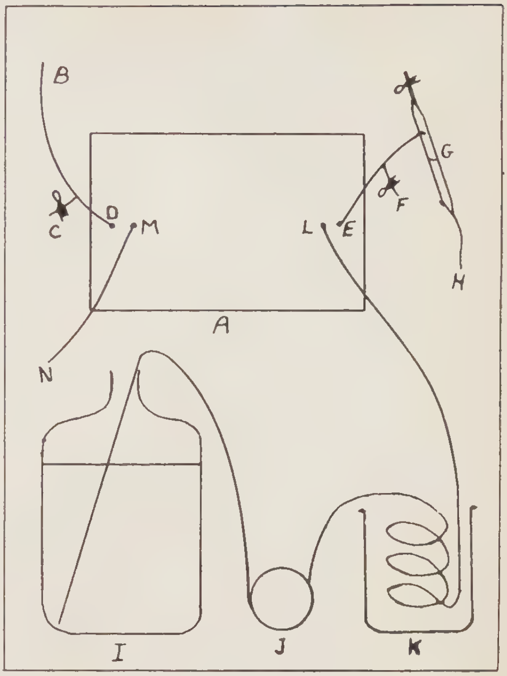

FIG. 2.
Diagram of connections of the artificial kidney to the animal and to the source of dialyzing solution. See text for explanation of letters.

The connections between "kidney" and animal are shown in Fig. 2. A rubber tube (B) sufficiently long to lead to the arterial cannula is connected to the blood spaces at one end of the "kidney." A similar tube (H) leads from the other end to the venous cannula. In this line is placed a simple glass air trap (G). It is also convenient to place glass T connections (C and F) in both arterial and venous lines. These are used to fill the "kidney" with blood and to administer intravenous solutions. During sterilization these 2 T tubes may be connected to the dialyzing solution inlet (L) and outlet (M). Steam at 5-15 lb. pressure is run through the arterial tube (B), the "kidney," glass trap (G), and the venous lines (H) for 30 minutes. Following sterilization the "kidney" is cooled and washed by running cold water for several hours through the dialyzing solution spaces only. Just before use the assembled "kidney" is rinsed by passing 6-10 liters of sterile saline through the blood spaces. We have found it convenient to place the kidney on an ordinary hospital bedside table and to reserve the lower part of the table for an apparatus designed to deliver dialyzing solution warmed to 37.5°C at any desired rate. This apparatus consists of a small centrifugal pump§ (J) controlled by a rheostat which propels dialyzing solution from a 5 gallon bottle (I) through a 20 foot length of ¼ inch diameter copper tube coiled in a thermostatically controlled

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§ Eastern Engineering Co., New Haven, Conn., Model B-1.

TABLE I.
Composition of Dialyzing Solution for the Dog.

Milliequivalents per l			
Na^+	148	Cl—	126
K^+	3	HCO_3=	24
Ca^{++}	5	HPO_4—	2
Mg^{++}	3	Lactate—	7
Total	159	Total	159

Glucose: 100 mg/100 ml
CO_2 gas to pH 7.35-7.45

Preparation of Concentrated Stock Solutions

Sol. A	G per l	Sol. B	G per l
KCl	9.0	$NaHCO_3$	84.0
$CaCl_2$	11.1	Na_2HPO_4	5.7
$MgCl_2 \cdot 6H_2O$	12.2	NaOH	9.6
NaCl	269.0		
Glucose	40.0		
Lactic acid	27.1 ml (assay 85-90%)		

500 ml each of solutions A and B are added to 19 liters of distilled water for use. The exact amount of lactic acid required to bring the pH of the diluted dialyzing solution to pH 7.3-7.5 is determined for each lot.

water bath (K), and then to the "kidney" at inlet tube (L). After passing through the "kidney," the dialyzing solution may be collected via tube (N).

The dialyzing solution is prepared to contain the electrolyte composition of the extracellular fluid of the normal dog with the addition of 100 mg of glucose per 100 ml. This composition is calculated from actual analyses of dog plasma obtained in this laboratory over the past 10 years and reported in part by Muntwyler *et al.*(18,19) Solution A (Table I) contains a small excess of lactic acid which upon mixing with the bicarbonate of solution B yields the required amount of dissolved CO_2 to maintain the pH at 7.35-7.45. This makes possible the presence of calcium, phosphate and bicarbonate in physiological concentrations without precipitation.

Twenty dogs have been dialyzed using the above apparatus. Morphine (0.5 mg/kg) or Merperidine Hydrochloride (Demerol) (50-150 mg) was injected subcutaneously and the femoral artery and vein exposed under local anesthesia (0.5% Pontocaine Hydrochloride). A plastic catheter 4 mm in diameter and 4-6 inches long is inserted through the femoral vein into the vena cava. Three mg of heparin per kg of body weight was injected intravenously through this catheter and the femoral artery cannulated. The "kidney" was then filled with heparinized donor blood, connected to the arterial and venous cannulae and blood allowed to flow through the dialyzer. At the end of the dialysis 15 ml of 1% protamine sulfate is slowly injected intravenously.[‖] This is usually adequate to restore the blood clotting time to a value of less than 15 minutes. In some animals an additional 50-100 mg of protamine sulfate were found necessary. The cannulae may then be removed. It is imperative that the clotting time be carefully watched if serious hemorrhages are to be avoided.

An arterial pressure of 100 mm Hg resulted in a blood flow of 150-250 ml per minute. Dialyzing solution flows at 400 cc per minute and is under a negligible pressure. The area of cellophane is 12,000 cm^2 which together with a total of 400 cc of blood in the "kidney" gives an average thickness of the film of blood of 0.66 mm. Under these conditions the clearance of urea from the dog's blood is usually about 50 ml of blood per minute (when calculated in a manner exactly analogous to the usual clinical method) and in occasional experiments has been as high as 60-80 ml per minute. It is of interest that creatinine dialyzes through cellophane at a rate only half as great as urea.

Most of the animals tolerated the dialysis without observable effects. Hemolysis did not occur. However, in a few cases the blood pressure decreased to as low as 30-50 mm but returned to normal within 1 to 3 hours. The cause of this effect has not been determined and is now under investigation.

In order to demonstrate the effects of treatment with the artificial kidney, dogs were given 5 mg per kg of anhydrous uranyl nitrate subcutaneously, a dose which is usually le-

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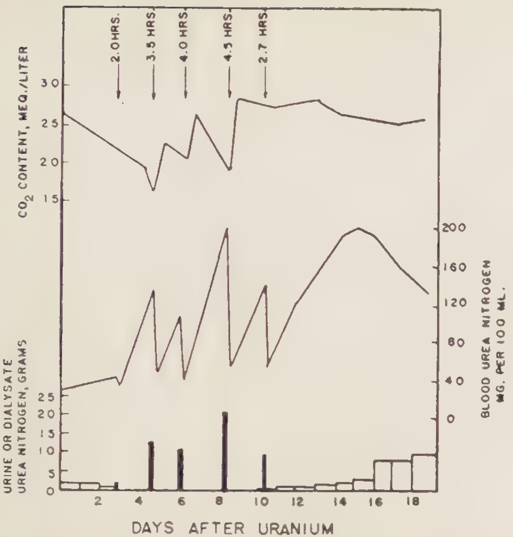

FIG. 3.

The effect of treatment with the artificial kidney on the blood urea nitrogen and the CO_2 content of plasma of a dog given a dose of uranium nitrate (5 mg per kg) which is usually lethal. The arrows represent dialyses of the durations indicated. Note the restoration of the blood urea nitrogen to almost normal levels and the elevation of the CO_2 content towards normal. The solid black bars at the bottom of the chart represent the urea nitrogen removed from the animal by the artificial kidney. This animal recovered.

thal.(20,21) The data on one of these animals are presented in Fig. 3. Three days after the injection of the uranium, the animal became anuric. At this time treatment was instituted and a total of 5 dialyses at 1-2 day intervals were successfully accomplished. The blood urea nitrogen was restored to nearly normal levels by each dialysis. A total of 57 g of urea nitrogen were recovered in the dialyzing solution. The ability of the artificial kidney to regulate acid-base balance is illustrated by the fact that the CO_2 content of the plasma which was decreased as a result of the inadequate renal function was repeatedly elevated toward normal levels. Chloride, sodium, and potassium remained within normal limits. Following the fifth dialysis, 10 days after the dose of uranium, the dog began to excrete urine and survived without further treatment.

Summary. An improved type of continuous dialyzer specifically designed for use as an artificial kidney has been described. A thin film of blood is made to flow between 2 sheets of cellophane supported between rubber pads which are grooved to allow passage of a dialyzing solution. Details of its operation are given and its application in the treatment of acute renal insufficiency in dogs is described.

Received Sept. 13, 1949. P.S.E.B.M., 1949, v72.

Intravenous Infusions of Concentrated Combined Fat Emulsions into Human Subjects.* (17492)

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The successful development of a method for the preparation of a 10% combined fat emulsion and the results after clinical investigation of this emulsion as reported by us warranted further studies in the preparation and administration of more concentrated fat emulsions.(1) The advantages to be derived from the more concentrated intravenous fat preparations, increased caloric potential in reduced volume, would constitute a further advance in the field of parenteral fat nutrition. The present report is devoted to the method of preparation of two combined fat emulsions with fat in 15 and 20% concentrations and a study of the effect of their intravenous infusion on human subjects.

Preparation of Concentrated Fat Emulsions.

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* Aided by a grant from the Ben Lewis Fund in Experimental Surgery.

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TABLE I.
Composition of 15% and 20% Concentrated Combined Fat Emulsions.

Conc. fat emulsion	% composition				G fat per liter	Calories per liter
	Coconut oil	Gelatin	Glucose	Amigen		
15%	15.5	2.20	5.4	3.6	155.0	1720
20%	20.2	2.66	5.5	2.2	202.0	2100

The successful preparation of the 15% and 20% combined fat emulsions was accomplished by adequate and thorough homogenization and emulsification procedures as described in our previous report on the 10% combined fat emulsion, whereby the homogenizer apparatus adapted to medical usage and the technic of manufacture of sterile emulsions were presented in detail.(1)

The constituents of the emulsions are solutions of 10% protein hydrolysate, 50% glucose, 6% intravenous gelatin (Knox-P-20) and refined coconut oils. All except the latter are available as sterile non-pyrogenic solutions in sealed flasks. The refined coconut oil is autoclaved before mixture with the other emulsion ingredients. In the present studies 15% and 20% concentrations of fatty oil were obtained in the preparation of the combined fat emulsions resulting in caloric values of approximately 1720 and 2100 calories respectively per liter of emulsion. The percentage composition of the various ingredients which make up the emulsions are listed in Table I with fat as the prime contributing factor to the increased caloric value of each preparation.

Intravenous gelatin was used exclusively as the emulsifying agent requiring between 2 and 3% for thorough emulsification of the 15% and 20% concentrations of fatty oil. The stability of both combined fat emulsions has been observed for a period of more than 7 months without "creaming" or "oiling out". The particle size of the emulsions was within the one micron range and active Brownian movement was exhibited on microscopic examination.

Method of Study. The combined fat emulsions were administered without selectivity to hospital patients who were being treated for a variety of surgical conditions. Several of these patients ran a chronic low grade fever. The studies were started in the morning with the subject in the postabsorptive state after a fasting period of 14 hours. Before the infusion was started a sample of blood was taken, the morning urine was obtained, and temperature, pulse, respirations and blood pressure were noted. The infusion was then given intravenously at a specific rate ranging from 20 to 80 drops per minute and varying in different patients. In every case the amount infused was 500 ml of either emulsion. In several subjects a second infusion was also given in the late afternoon. During the course of the infusion and throughout the post-infusion phase until the next morning, blood and urine samples were taken and the above mentioned clinical notations were made at regular intervals. The following tests were made routinely on the blood; hemoglobin, red cell count, white cell count with differential, sedimentation time, chylomicron count and specific gravity. Clinically, subjective and objective constitutional reactions relative to the infusion were studied. X-rays of the lungs were taken in series for any lesions due to fatty infiltration, pulmonary irritation or pulmonary emboli. Final check examinations were made 2 weeks after completion of the infusions for hemolytic anemia and for possible liver or kidney damage. Surgical liver and lung biopsies were obtained from several subjects who received the emulsion. These specimens were prepared for microscopic analysis by staining with osmic acid, Sudan IV and hematoxylin-eosin.

Results. The 15% and 20% combined fat emulsions were administered intravenously into 25 human subjects for a total of 51 infusion studies. Details relative to the method of administration, volume infused, temperature response and constitutional reactions are supplied in Table II. Temperature changes were computed according to the degree of

TABLE II.
Immediate Effects from Infusions of Concentrated Fat Emulsions into Human Subjects.

Emulsion	Volumes infused (ml)	g fat infused	No. of patients infused	No. of infusions given	Reactions.					
					Temperature rise					
					<1°	1°-1.9°	2°-2.9°	>3°	Chill	Vomiting
15%	500 to 600	77.5 to 93.0	14	23	8	3	5	7	7	2
	700 to 900	108.5 to 139.5	2	9	3	0	3	3	2	0
	1000	155.0	2	7	1	1	2	3	1	0
20%	500 to 600	101.0 to 121.2	7	12	4	4	3	1	4	1
Totals			25	51	16	8	13	14	14	3

elevation above the initial pre-infusion temperature but were not corrected for changes due to the disease of the patient. The incidence of chills totalled 14 of all infusions given. Of the latter the occurrence of 7 cases of chills were correlative with high temperature and the administration of the emulsion at the rate of 80 drops per minute. Upon reduction of the rate of infusion these same subjects on other occasions did not repeat the chill when re-infused with the fat emulsion. Chills were the severest type of reaction encountered in the present series. Upon the administration of 50 mg of either pyribenzamine or benadryl the latter reaction was considerably reduced. Vomiting, headache and dizziness occurred in 4 instances and were in association with a chill. Other reactions such as coughs, allergies and fatty taste did not occur in this series. There were also no fatalities. (Table II).

The values for hemoglobin, hematocrit cell volume and red and white cells tended to fall below their initial values during the infusion phase of the emulsion and returned to their original levels shortly before or after the termination of the infusion. The erythrocyte sedimentation rate showed an increase in rapidity of sedimentation which average 25% to 35% greater than the pre-infusion sedimentation time. It also returned to its initial level. The differential white count was not indicative of any trend. The specific gravities of blood and plasma showed a temporary dilution effect due to the infusions followed by a return to normal limits (Table III).

Changes of blood pressure in response to the injection were variable. During the infusion period there was generally a slight elevation of blood pressure followed by a moderate fall which persisted for 2 hours after completion of the infusion. The elevation of systolic pressure did not exceed 20 mm Hg and the fall in systolic pressure ranged from 10 mm to 25 mm Hg. The diastolic pressure tended to fall towards the end of the infusion but also returned to its normal level. There were no falls in blood pressure which indicated any tendencies toward the development of shock.

Counts of chylomicrons were made from peripheral venous blood under dark field illumination with a net micrometer in the eyepiece of the microscope. In the post absorptive state the pre-infusion count varied from zero to 10 chylomicrons. After the infusion was started there was a rapid rise in the number of chylomicrons to a peak. At about the mid-point of the infusion the chylomicrons began to decrease in number and return slowly to the initial level.

Consecutive urine studies were made in 25 infusion tests. In no case was a positive test for blood or urobilinogen obtained which was attributable to the fat emulsion. Chemical tests for fat in the urine were negative. Dark field examination of the urine for chylo-

TABLE III.

Effects of Intravenous Infusions of Combined Fat Emulsions into 2 Subjects. A—Received 15% combined fat emulsion. B—Received 20% Combined fat emulsion.

Time (min.)	Amount infused	Subject	Temp.	Pulse	Blood pressure	Hemoglobin	R.B.C. count (million)	W.B.C. count (thousand)	Hematocrit	Sedimentation time	Chylomicron count	Urine acetone
0	0	A	98.4	60	130/80	13.5	3.95	5.8	42	26	12	neg.
		B	98.8	74	160/92	14.6	4.75	7.0	46	23	0	,,
60	100	A	98.4	60	130/80	14.0	4.20	6.2	42	23	150	neg.
		B	99.0	78	168/92	14.8	4.75	6.8	41	23	100	,,
120	225	A	98.6	68	154/78	15.0	3.80	6.4	40	38	350	1+
		B	99.0	80	184/88	14.6	4.68	5.0	39	34	400	neg.
180	350	A	99.0	76	130/88	14.0	3.70	6.4	38	41	300	1+
		B	100.0	86	186/74	14.0	4.70	5.6	41	34	400	2+
240	500	A	102.4	100	126/84	14.5	3.65	6.6	41	45	260	3+
		B	102.0	104	178/78	13.8	4.90	5.7	45	34	200	1+
360	—	A	100.6	84	130/88	14.5	3.68	5.8	43	27	100	neg.
		B	102.6	106	182/90	14.0	4.10	6.0	46	30	80	1+
480	—	A	100.0	100	156/70	14.5	3.95	5.6	43	30	100	neg.
		B	102.0	94	188/74	14.2	5.00	6.6	40	28	50	,,
600	—	A	100.4	96	132/88	15.0	3.45	6.0	42	36	20	neg.
		B	100.0	92	160/80	14.4	4.92	6.8	46	30	60	,,
720	—	A	100.0	88	110/80	14.8	3.90	6.2	40	34	50	neg.
		B	98.4	84	174/88	14.6	4.80	7.4	48	28	0	,,
24 hr	—	A	98.8	72	116/80	14.8	3.95	5.2	43	30	0	neg.
			98.6		178/96	13.8	5.00	7.8	40	27	8	,,

microns showed a moderate elevation in the number of fat particles. In 4 infusion studies with the 15% fat emulsion and in 3 with the 20% fat emulsion, positive reactions were obtained for acetone varying from 1+ to 2+ in 2 or 3 of the intermediate urine specimens examined during the course of the infusion. During the post-infusion phase the tests for acetone occasionally showed a faint trace and remained negative thereafter. There were no positive tests for diacetic acid. There were no indications by these tests of any greater acetone spillage occurring in recipients of the 20% fat emulsion as compared with recipients of the 15% fat emulsion.

Follow-up studies for hematologic complications in subjects who received either single or multiple infusions showed no signs of hemolytic anemia. Likewise x-rays of the lungs gave no evidence of fatty emboli or pulmonary irritation. Liver and lung biopsy specimens failed to show any organic lesions which could be attributed to the intravenous infusion of fat. Liver and kidney function studies showed no pathologic alterations which were attributable to the emulsions.

Comment. The effects of the intravenous infusion of either the 15% or 20% combined fat emulsions were in many respects similar to those noted in our clinical studies on the infusion of the 10% combined fat emulsion.(1) These pertained particularly to hematologic changes, blood pressure response and follow-up observations on liver, lung and kidney function. Constitutional reactions such as high temperatures or chills averaged 27% as compared with 9% with the more dilute fat emulsion. About 50% of the reactions encountered in this series were found to be due to the speed with which the emulsion was administered, the fast infusions being associated with the chill reaction. The temperature elevations could be accounted for on the basis of a sudden plethora of fat. In any case the concentrated fat emulsions required cautious careful administration.

Summary. Two types of combined fat emulsions, one containing 15% fat and the other 20% fat, were infused into 25 human subjects. A total of 51 of these concentrated fat emulsions were administered intravenously. The incidence of constitutional reactions was 27%. A variety of laboratory and clinical observations were made in conjunction with these infusions.

Received Sept. 23, 1949. P.S.E.B.M., 1949, v72.

Malignant Lymphoid Tumors in Orchidectomized Mice Receiving Hypophyseal and Ovarian Grafts at Various Ages.* (17493)

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While studying the effects of anterior hypophyseal and ovarian transplants on mammary growth in male castrate mice of strain A, we noted the occurrence of lymphoid leukemia and lymphosarcoma in a number of animals bearing these grafts.(1) The present report deals with the incidence of these tumors in the various experimental groups.

Material and methods. The experiments have been described in detail in a previous paper.(1) Briefly, 161 male mice of the closely inbred strain A kept on a standard diet of Purina Laboratory Chow and water were castrated at the age of 3 weeks. The animals were divided into two age groups: *I. Younger age group* grafted at the age of one month: Twenty-eight castrates received four anterior hypophyses each; 26 received 2 or 4 ovaries each; 25 received a combination of 4 hypophyses and ovaries. *II. Older age group* grafted at the age of 7 months: Twenty-eight

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* The investigation was supported by a research grant from the National Cancer Institute, United States Public Health Service.

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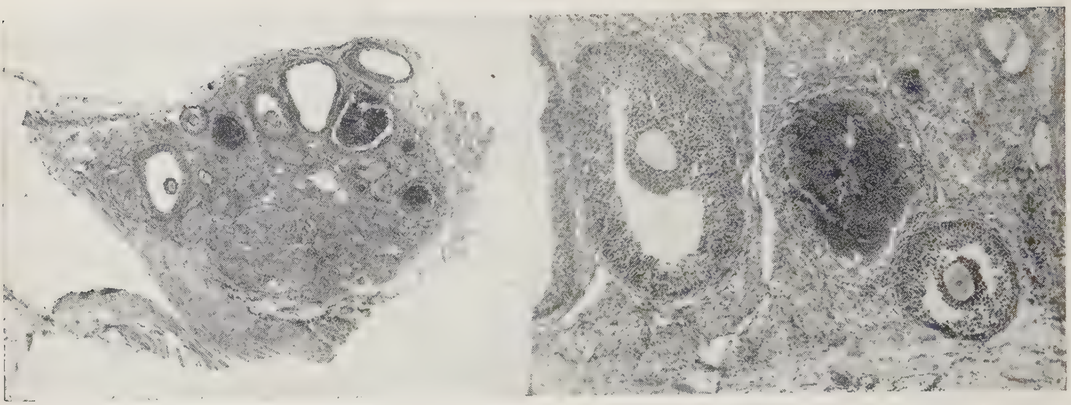

Fig. 1 and 2.
Section through an ovarian graft 21 months after transplantation. Fig. 1 (left) magnification 70×. Fig. 2 (right) magnification 345×.

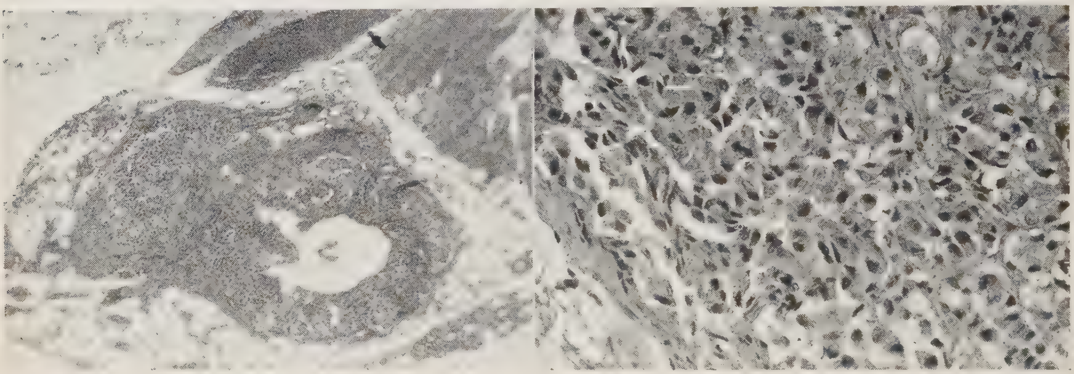

Fig. 3 and 4.
Section through an hypophyseal graft 14 months after transplantation. Fig. 3 (left) magnification 25×. Fig. 4 (right) magnification 93×.

castrates received anterior hypophyses; 25 received ovaries; and 29 received a combination of 4 hypophyses and ovaries. All grafts were obtained from freshly killed animals closely related to the host (syngenesiotransplants).(2) The glands were transplanted into a subcutaneous pocket of the chest wall.

Observations. In untreated mice of strain A which is known to have a 3.8% incidence(3) of spontaneous lymphoma only an occasional case of malignant lymphoid tumor was noted. These tumors with or without leukemia manifested themselves in large neoplasms of the mediastinum or mesentery, or in diffuse infiltration of lymph nodes, spleen, liver, kidney, lung or heart, leukemic blood picture, or in a combination of several of these findings. Only those cases were classified as malignant lymphoma in which gross and microscopic changes in liver, kidney, lung, heart, and frequently also in blood smears were sufficiently advanced to permit a clear cut diagnosis. Cases in which the microscopic findings were merely suggestive of lymphoma but not definite were listed as preleukemic. Since the present experiments were originally not designed to include observations on leukemia, the bioassay of suspected cases of malignant lymphoid disease, which would have been desirable, was not made. Mice showing merely hemopoiesis were grouped among the negatives. Microscopic examination showed

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TABLE I.
Incidence of Malignant Lymphoid Tumors and Time of Appearance of the Neoplasms in the Various Experimental Groups.

Type of graft	Age at grafting (mo.)	No. of mice	Age at death of all mice (mo.)		Animals with lymphoma				Animals with preleukemia change			
							Age (mo.) of appearance				Age (mo.) of appearance	
			Mean	Range	No. of cases	%	Mean	Range	No. of cases	%	Mean	Range
I. Hypophysis	1	28	16.4	9–20	7	25.0	16.6	9–20	3	10.7	17.3	16–18
II. Ovaries	1	26	18.2	9–24	7	26.9	20.0	15–24	3	11.5	20.0	13–24
III. Hypophysis and ovaries	1	25	17.4	9–22	9	36.0	18.7	12–21	2	8.0	19.5	18–21
IV. Hypophysis	7	28	19.3	12–25	3	10.7	22.0	19–24	3	10.7	20.7	18–22
V. Ovaries	7	25	16.8	12–26	2	8.0	15.0	12–18	4	16.0	16.5	12–23
VI. Hypophysis and ovaries	7	29	15.7	12-24	4	13.8	17.5	12-24	3	10.3	17.7	14–22

that grafts may remain alive for a considerable length of time. The following photomicrographs 1-4 demonstrate well preserved hypophyseal and ovarian grafts. As had been found in regard to mammary cancers in these animals,(1) the development of malignant lymphoid tumors could not always be correlated to the state of preservation of these transplants at the time of necropsy. It must thus be assumed that grafts may exert an effect for some time and then undergo regression.

The incidences of malignant lymphoma, the mean age at death, and the age range at death in the various experimental groups are given in Table I.

Younger are group: Transplants of anterior hypophysis had a leukemogenic effect of about the same order (25%) as ovarian grafts (26.9%). However, ovaries were slower in calling forth this result than were hypophyseal transplants, the mean age at death of animals of the former series being 20 months as compared with 16.6 months in the latter group. Anterior hypophysis grafted together with ovaries produced malignant lymphoma about 40% more often, namely in 36% of the animals, than either hypophysis or ovaries alone, and the mean age at death in this group was 18.7 months.

Older age group: 10.7% of the castrates bearing transplants of anterior hypophysis had lymphomas at the mean age of 22 months; 8% of the animals bearing ovarian grafts alone developed lymphoid tumors at the age of 15 months, and neoplasms were observed in 13.8% of the mice bearing grafts of anterior hypophysis together with ovaries. In this group, the mean age at death was 17.5 months.

Discussion. In castrate male mice of strain A bearing anterior hypophyseal grafts, malignant lymphoid tumors were found to develop in increased numbers. This leukemogenic effect of anterior hypophysis was apparently not mediated through the ovaries since it was present in castrates which did not carry ovarian grafts. The mode of action of the anterior hypophyseal hormone in this type of leukemogenesis is unknown. Tentatively, however, the following explanation may be offered: Anterior hypophyseal hormone by stimulating the adrenal cortex may cause a prolonged depletion and atrophy of the lymphoid tissue. This atrophy may become a stimulus to or may represent a step in leukemogenesis,(4-8) since malignant lymphoma is often preceded by atrophy of lymphoid tissue. The leukemogenic effect of the anterior hypophyseal hormone is probably not due to increased output of estrogen by the adrenals, because the mammary glands of these castrates failed to develop cancer or marked acinar proliferation in contrast to conditions found in the castrates carrying ovarian grafts.(1) As to the formation of lymphomas by transplanted ovaries, the results are in agreement with those obtained by the administration of estrogens in mice of various ages,(9) and may be attributed to the production of such hormones by the ovarian grafts. The increased incidence and the shortened latent period of lymphoma in mice bearing hypophyseal grafts together with ovarian transplants seem to represent a cumulative effect of the two hormones given off by the grafts. Whether or not the mechanism by which estrogens produce lymphoid tumors is in any way connected with that initiated by anterior hypophyseal hormone or whether two different mechanisms are involved, is unknown.

In the older age group, the incidence of lymphomas induced by anterior hypophysis fell to 10.7%. Thus the susceptibility to these neoplasms declined somewhat, as the age of the animal at the time of grafting advanced. However, the difference in the mean lymphoma age in the two age groups (5.4 months) agrees closely with the difference in age at the time when the transplants were made (6 months' difference). The latent periods of tumor formation were thus similar in both series. Of the castrates of the older age group carrying ovarian grafts 8% developed lymphoid tumors, and of those bearing ovaries together with anterior hypophysis 13.8% had these neoplasms. However, these tumor incidences are probably not truly representative of the effects of these two types of grafts in the older age group: There was a decrease in the lymphoma incidence and a lowering of the lymphoma age, whereas one would expect the lower lymphoma incidence to be associated with a prolongation of the latent period. This apparent discrepancy may, however, be explained by the relatively early age at death of the mice in these two groups. The total incidence of malignant lymphoid tumors and the mean lymphoma age would probably have been higher if the animals of these groups had reached an older age. There was no obvious cause for the relatively short life spans of these mice particularly since none of them developed mammary cancer. As to the possible role of an age factor it may be stated: However small the incidence of induced lymphoma in the older age groups, it was still larger than the zero incidence of mammary cancers observed in the same mice.

Conclusions. Mice of strain A orchidectomized before weaning and bearing grafts of anterior hypophysis, of ovaries, or of anterior hypophysis together with ovaries from the age of one month on, developed malignant lymphoid tumors. Anterior hypophysis seems to exert its leukemogenic effect (1) through the ovaries and (2) independently of the grafted ovaries, possibly through the adrenal cortex. Transplantation of ovaries together with anterior hypophysis resulted in the highest incidence of malignant lymphomas. The age factor plays in this type of leukemogenesis a less important role than in the production of mammary cancers by anterior hypophyseal and ovarian grafts.

Received Sept. 27, 1949. P.S.E.B.M., 1949, v72.

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Voluntarily Ingested Sodium Chloride as a Lymphagogue in the Rat.*†

(17494)

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In the course of studies on factors influencing the rate of lymph flow, experiments were carried out to determine the effect of fasting, feeding, and alterations in electrolyte intake on the formation of thoracic duct lymph in the unanaesthetized rat. The results of these experiments indicate that the ingestion of 1% NaCl solution by the fed animal causes an increase in rate of lymph flow which is out of proportion to that seen in control animals.

Methods and Materials. Male rats of the Long-Evans strain maintained under comparable environmental conditions were prepared for lymph collection by the method of Bollman, Cain and Grindlay.(1) This technique involved the insertion of a plastic cannula into the thoracic duct immediately caudal to the left crus of the diaphragm, at which point the cannula was tied in place, the delivery end being drawn out through the abdominal wall. In these experiments, a ventral midline abdominal operative approach was employed. The rat was then placed in a cage(2) which permitted *ad libitum* food and fluid intake, but which restrained activity to the point of allowing collection of lymph samples in the unanaesthetized state. The animals were anaesthetized for the preparation of the fistula by means of intraperitoneal administration of a 2% aqueous solution of sodium pentobarbital in the amount of 7 mg/100 g body weight. Following recovery from the anaesthetized state, the animals were placed on free oral intake of the regular laboratory diet (XIV) or were fasted as indicated. Each group of animals, as indicated in the tables, was given access to tap water or 1% NaCl for drinking purposes.

Results. The experimental results are presented in Tables I and II. Table I presents the results of experiments showing the rate of lymph flow in the fasting and fed animals with free access to water, as compared with the rate of lymph flow in the fasted and fed animal given 1% NaCl for fluid intake, and, finally, the effect of alternation for 24-hour periods of water and 1% NaCl in the fed animal. It will be noted that lymph flow is least in the group of animals fasted and given access to water. Animals fed, with access to water, or fasted with oral intake of 1% NaCl produced comparable amounts of lymph as expressed in ml of lymph per hour per 100 g body weight. On the other hand, rats given access to both food and 1% NaCl for drinking purposes markedly increase their lymph flow to an amount 5-6 times the volume of that of the fed animal on water. The difference in rate of lymph flow is seen most markedly in the last group of animals in this table in which water and 1% NaCl were alternated for 24-hour periods. The output of lymph during the period on saline solution is markedly higher than is the output during water ingestion.

The increase in lymph flow produced by oral intake of 1% NaCl increases with duration of the experiment. Table II presents the average daily lymph volumes in a group of rats maintained on water and 1% NaCl in the fed state. It will be noted that the lymph flow in the animals maintained on water is maximal on the second day and falls off gradually, whereas the flow of lymph in the animals maintained on NaCl increases daily to an amount which exceeds 50% of the body weight of the animal. In one in-

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* Assisted by grants from the Board of Research of the University of California and the National Heart Institute of the United States Public Health Service.

† A portion of the experimental data was presented at the meeting of the American Association of Anatomists at Philadelphia in April, 1949.

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TABLE I.
Effect of Varying Food and Fluid Intake on Rate of Lymph Flow.

Diet	Fluid intake	No. of rats	Avg BW (g)	Time of collection, (hr)	Lymph flow (ml)	Rate of flow (ml/hr/100 g)
Fasting	Water	4	160* (110-250)	29* (18-47)	11.4* (7.3-18.6)	0.26* (0.12-0.38)
,,	1% NaCl	6	250 (145-320)	25.5 (22-30)	29 (15-61)	0.48 (0.16-0.98)
Fed	Water	4	229 (212-240)	105 (93-118)	106 (64-163)	0.44 (0.29-0.62)
,,	1% NaCl	3	280 (170-315)	124 (111-140)	727 (657-784)	2.45 (1.98-3.29)
,,	1% NaCl	9	239 (183-386)	52 (40-72)	165 (137-302)	1.45 (0.88-2.2)
Fed†	Water	5	153 (130-168)	43 (20-72)	60 (13-123)	0.86 (0.39-1.21)
	1% NaCl			44 (27-72)	216 (57-457)	3.3 (1.5-4.7)

* Average value for the group. Figures in parentheses indicate ranges.

† The animals in this group were maintained alternately for 24 hr periods on 1% NaCl solution and water *ad lib.*

TABLE II.
Average Daily Output of Lymph in Fed Rats Maintained on Orally Ingested Water or 1% NaCl Solution.

Fluid intake	No. of rats	Avg BW (g)	Avg vol. (ml) of lymph per consecutive day				
			1	2	3	4	5
Water	4	229	9	31	27	21	18
1% NaCl	3	280	28	76	125	138	186

stance (not recorded in table) 302 cc of lymph was collected in 23 hours from a rat weighing 145 g.

Discussion. The experiments reported here indicate that the fluid intake of the rat rises remarkably in the presence of a lymph fistula, if the animal ingests added sodium chloride. The control rat with a thoracic duct fistula is apparently able to make up for the loss in nutrients and electrolytes by establishing a dietary and fluid intake which is satisfactory for the maintenance of a temporary equilibrium. If, however, sodium chloride is added to the diet, the requirement for fluid intake rises considerably. The abrupt increase in intake of saline drinking solution suggests that the animal is vigorously attempting to repair loss of fluids and electrolytes via the lymph fistula.

It is well known that the intravenous injection of large amounts of crystalloids will produce an increased lymph flow from the thoracic duct. The explanation for this effect(3) is that an hydremic plethora results, with consequent lowering of plasma colloidal osmotic pressure and increased capillary hydrostatic pressure. There is, then, a resultant loss of fluid to the tissue spaces, with consequent increase in lymph flow. This does not, however, explain the reason for the greatly increased *voluntary intake* of 1% NaCl solution and resultant lymph output as seen in the experiments cited here.

Summary. 1. A comparison is made of the

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rates of thoracic duct lymph flow in unanaesthetized male rats maintained under the experimental conditions of fasting and feeding. with free access to either water or 1% NaCl solution.

2. Thoracic duct lymph flow is at a low level in the fasted animal with free access to water. The rate of flow is increased by feeding or access to 1% NaCl solution. The rate of flow in the *fed* animal with free access to 1% NaCl is increased appreciably over that of the controls, and out of proportion to any hitherto employed method for *voluntarily* increasing lymph flow in the otherwise normal animal. In some instances, the lymph flow of the NaCl treated animal exceeded the body weight of the animal in a 24-hour period.

3. Alternation of periods of access to water and saline solution produced corresponding low and high rates of lymph flow in the fed animals.

Received Sept. 27, 1949. P.S.E.B.M., 1949, v72.

Specificity of Paper Partition Chromatography for Analysis of Free Amino Acids in Unhydrolyzed Urine.* (17495)

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The paper partition chromatographic technique devised by Consden, Gordon, and Martin(1) may be more specific for detecting free amino acids in urine than microbiological methods. The number of amino acids found by microbiological assay in unhydrolyzed urine excreted by normal human subjects is greater than the number detected by paper partition chromatography.

Paper partition chromatography has been used to investigate the amino acids excreted in the urine by normal subjects and selected cancer patients. Aliquots of a 24-hour urine collection or of pooled samples obtained over a 5 or 6 day period were standardized by Kjeldahl analysis to contain either 10 mg of total nitrogen or by the Van Slyke ninhydrin method(13,14) to contain 0.5 mg of α-amino nitrogen per milliliter. All samples were stored at 3°C under toluene. Aliquots requiring concentration were lyophilized and re-diluted to the desired volume. Other aliquots were hydrolyzed by boiling with 50% hydrochloric acid for 24 hours. After removal of the acid under reduced pressure, the hydrolysate was also standardized to contain 10 mg of total nitrogen per milliliter.

Standardization of the urine permits comparison of urine specimens from period to period and from patient to patient. The concentration selected yields chromatograms of satisfactory color intensity and insures detection of most of the amino acids present in the specimen. Concentration of normal urines to contain more than 40 mg of total nitrogen per milliliter prevents adequate separation since salts and other substances interfere. Removal of salt by common ion effect precipitation, impregnation of the filter paper and saturation of the solvents with salt has been unsatisfactory.

A two-dimensional chromatogram on Whatman No. 1 filter paper was prepared from each of the samples(1). One hundred microliters of the standardized solution were applied to the paper, and 25 microliters of 30% hydrogen peroxide were superimposed on the spot to effect quantitative oxidation of cystine and methionine to cysteic acid and

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* Funds to support this investigation were provided by a grant from the Donner Foundation, Inc.

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methionine sulfone, respectively, which appear as discrete spots on the developed chromatogram. Without such conversion, cystine is destroyed and methionine is obscured by leucine, isoleucine, tryptophane, and phenylalanine. Phenol and a mixture of equal parts of γ-collidine and 2,4-lutidine were the solvents regularly employed.

Each spot on the chromatogram was outlined in pencil as soon as possible after color had been developed with 0.1% triketohydrindene hydrate (ninhydrin) in n-butanol to insure that interpretation was complete before the color faded. The amino acids and peptides were identified after calculation of the R_F values, by comparison with a control sheet to which standardized solutions of the amino acids had been applied, or by reference to a map of the spots similar to the one prepared by Dent.(3)

Other ninhydrin-reacting substances than α-amino acids(7) have seldom been found in urine in sufficient concentration to cause confusion or interference. The identity of the spots which comprise the chromatogram is frequently verified to insure that known spots are not masking the presence of other substances. Glycine, for example, may completely obscure asparagine, and β-alanine may conceal glutamine. Visual identification of certain spots may be difficult because of their close proximity and identical color as in the case of leucine, isoleucine, phenylalanine, and tryptophane.

Chromatograms of standardized unhydrolyzed urines from a number of normal subjects generally reveal the presence of alanine, cystine, glutamine, glycine, histidine, and serine. Three-fold concentration of an aliquot has revealed only β-alanine, leucine, threonine, tyrosine, and valine in addition. Although glutamic acid may be present, it has been demonstrated in this laboratory that it represents, in part at least, the amide which has undergone hydrolysis during storage or when exposed to the solvents during the run. Glutathione is also seen, as well as "over-glycine", often called taurine in the literature.(3) Doubt exists that "over-glycine" is in fact taurine, however. The infra-red absorption spectrum obtained from a sample of "over-glycine" washed from undeveloped chromatograms after localization differs from that of a standardized taurine solution.(5)

Dent, utilizing the same technique, has reported that normal urines usually contain glycine and alanine.(2) Glutamic acid and valine occasionally appear, and α-amino-n-butyric acid, citrulline, histidine, leucine, serine, and tyrosine may be found. His specimens, however, were not standardized to constant nitrogen concentration and usually contained less α-amino nitrogen than the samples analyzed in this investigation.

Arginine, cystine, glycine, histidine, leucine, methionine, phenylalanine, serine, threonine, tryptophane, tyrosine, and valine are found regularly by microbiological assay in an allegedly uncombined state in the urine of normal human subjects.(4,11,12,15) Proline, lysine, and isoleucine are sometimes present, too. Aspartic acid is detected infrequently. Free glutamic acid is found only when precautions have been inadequate for preservation of the specimens.(4) The amino acids detected in normal, unhydrolyzed urines by both methods are compared in Table I.

Determination of the amino acids in hydrolyzed urines by both microbiological and chromatographic methods yields comparable results. Whether measured microbiologically or chromatographically, hydrolysis results in an increase in the number and amount of amino acids over those found in the free state.(4,11,12,15,16)

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TABLE I.
Free Amino Acids in Unhydrolyzed Urine.

	Methods		
	Paper partition chromatography		
Amino acid	10 mg total N per ml	34 mg total N per ml	Microbiological assay (4, 11, 12, 15)
—alanine	+	+	0
’’	—	+	0
Arginine	—	—	+
Cystine	+	+	+
Glutamine	+	+	—
Glycine	+	+	+
Histidine	+	+	+
Isoleucine	—	*	+
Leucine	—	+	+
Lysine	—	—	+
Methionine	—	—	+
Phenylalanine	—	*	+
Proline	—	—	+
Serine	+	+	+
Threonine	—	+	+
Tryptophane	—	*	+
Tyrosine	—	+	+
Valine	—	+	+
Total No.	6	11	15

* May be obscured by leucine.
+ Reported as usually present.
— Not reported as usually present.
0 No information available.

Certain of the amino acids are excreted mainly in the combined form. Ninety-nine percent of aspartic acid, 90% of glutamic acid, 80% of proline, 77% of valine, and 71% of isoleucine, for example, are known to be excreted in the urine in peptide combination or as conjugates.(15) More lysine, threonine, leucine, and arginine have also been found to be excreted in the bound form than in the free state.(12) Harvey and Horwitt(6) concluded that not all of the free amino acid determinations by microbiological assay yield valid results because of the inhibiting effect of urea and other substances in urine.

As the sensitivities of paper partition chromatography and microbiological assay are comparable,(3,8,9) the discrepancy in results suggests that the microbiological procedure may detect other than free amino acids in urine. Peptides or similar substances excreted in urine may not yield a color after reaction with ninhydrin and hence are not detectable on chromatograms, but their component amino acids apparently are available to the micro-organisms. The growth-promoting action of certain peptides has been demonstrated with some strains of micro-organisms,(10) and utilization by other strains may yet be demonstrated.

Summary The number of free amino acids found in unhydrolyzed urine by paper partition chromatography is less than that indicated by microbiological assay. No discrepancy is apparent between the methods when hydrolyzed urines are analyzed. As the sensitivities of the two methods are comparable, it is suggested that microbiological techniques are not as specific as the chromatographic procedure for the detection of urinary amino acids in the uncombined state.

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Received October 4, 1949. P.S.E.B.M., 1949, v72.

Biotin in the Bacterial Deamination of Certain Amino Acids. (17496)

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Lichstein and coworkers(1,2,3,4) have reported that the aspartic acid, serine, and threonine deaminase activity of certain bacteria may be inactivated by exposure of the cells for a short time to pH 4 in M phosphate buffer. In their experiments the deaminase activity of such inactivated cells could be restored with biotin. Subsequent investigators(5) have been unable with biotin to restore deaminase activity to bacterial cells similary treated and the fundamental observation that biotin somehow functions in deaminase activity has remained unconfirmed by any other group. Although certain discrepancies exist between the results obtained by Lichstein and those found in this laboratory, the activation of treated cells with biotin under certain defined conditions has been obtained, thus furnishing corroborative evidence for the involvement of biotin in the bacterial deamination of certain amino acids.

Procedure. Eighteen-hour cultures of the organisms used were obtained following growth at 37°C on a medium composed of 1% yeast extract (Difco), 1% tryptone (Difco), 0.5% disodium hydrogen phosphate, and 0.1% sodium formate. The medium was dispensed in 100 ml quantities in 250 ml Erlenmeyer flasks. The pH before autoclaving at 120°C for 15 minutes was 7.35. Following growth the organisms were centrifuged down rapidly (Servall angle centrifuge, plastic cups), resuspended in 50 ml of distilled water, and recentrifuged. The washed cells thus obtained were suspended in about 3 ml of M phosphate buffer of pH 4. Inactivation with respect to deaminase activity was accomplished on aliquots of this suspension. These aliquots were dispensed in open test tubes that usually were immersed in a water bath at 37°C. Thirty minutes was the usual inactivation time. Immediately following inactivation of the cells the deaminase reaction was carried out at 37°C by appropriate additions to the same tubes. A protocol for deamination at pH 7 is given in Table I. When the deamination reaction was carried out at pH 4, M phosphate buffer of pH 4 replaced the Na_3PO_4 solution and was used also as a solvent for the aspartic acid employed.

Following the deamination reaction at either pH enzymatic activity was stopped by the addition of 0.5 ml of 20% trichloroacetic acid to each tube. The tubes then were centrifuged until a completely clear supernatant was obtained. Ammonia formed in each tube was determined by adding to 2 ml of supernatant 2 ml of 10% NaOH and 1 ml of Nessler's reagent (gum ghatti stabilized). The color intensities were read in a Klett-Summerson colorimeter against a series of similarly prepared standard tubes of ammonium sulphate. The ammonia nitrogen found in tube 1 was subtracted from the results obtained in each other tube to give the ammonia nitrogen originating from the deamination of aspartic acid by the bacterial preparations used.

Results. The results obtained in a series of experiments are summarized in Table II. These data confirm those of Lichstein to the extent that they show that cells of *Proteus vulgaris* or *Escherichia coli* can be obtained with a reduced ability to deaminate aspartic acid and that when the deaminase reaction is run at pH 7, after inactivation of the cells at pH 4 in M phosphate buffer, activity can be restored by either biotin or yeast extract. Attempts at reactivating cells with biotin when the deaminase reaction was run at pH 4 in M/2 phosphate buffer, such as described by Lichstein in his paper(3) demonstrating special activity for yeast extract, have been

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TABLE I.
Protocol for Aspartic Acid Deaminase.

	No. 1, ml	No. 2, ml	No. 3, ml	No. 4, ml
Bacterial suspension in pH 4 M phosphate buffer	0.5	0.5	0.5	0.5
Na_3PO_4 (saturated)	0.5	—	—	—
Aspartic acid* in Na_3PO_4 (saturated)	—	0.5	0.5	0.5
H_2O	1.0	1.0	—	—
Biotin†	—	—	1.0	—
Yeast extract‡	—	—	—	1.0

* 266 mg dl-aspartic acid dissolved in 100 ml saturated Na_3PO_4. Calculated to give 0.005 M dl-aspartic acid in the final reaction mixture.

† 0.0001 γ or 0.001 γ d-biotin/ml.

‡ 0.1 mg or 1.0 mg yeast extract/ml.

TABLE II.
Summary of Aspartic Acid Deaminase Experiments.

Exp. No.	Organism*	pH of deaminase reaction	Ammonia nitrogen formed		
			No supplement, γ	Biotin, γ	Yeast extr., γ†
1	*Proteus vulgaris*	7	11.7	15.8 (0.001 γ)	15.1 (1 mg)
2	” ”	7	7.0	14.8 (0.001 γ)	13.7 (1 mg)
3	” ”	7	4.3	15.9 (0.001 γ)	17.3 (1 mg)
4	” ”	7	2.3	11.3 (0.0001 γ)	2.3 (0.1 mg)
5	” ”	7	2.3	16.1 (0.0001 γ)	5.4 (0.1 mg)
6	” ”	4	0.0	0.0 (0.001 γ)	0.0 (1 mg)
7	” ”	4	0.6	0.6 (0.001 γ)	0.0 (1 mg)
8	” ”	4	0.1	0.5 (0.0001 γ)	1.0 (0.1 mg)
9	*Escherichia coli* (Gratia)	4	0.0	0.0 (0.001 γ)	0.0 (1.4 mg)
10	*Escherichia coli* (Gratia)	4	3.0	3.0 (0.001 γ)	0.0 (1.4 mg)

* All bacterial preparations were washed with water and inactivated by exposure for 30 minutes at 37°C to M phosphate buffer of pH 4 except in experiments 7, 9 and 10, where inactivation was at 25°C, and in experiment 10, where in addition to inactivation at the lower temperature the cells also were unwashed.

† 1 mg of yeast extract contains after vigorous acid hydrolysis prior to microbiological assay about 0.001 γ of biotin.

unsuccessful. When the amounts of biotin and yeast extract used to stimulate deamination at pH 7 are titrated downward, contrary to Lichstein who worked at pH 4, biotin was found to be more active in the system than is an equivalent of biotin as it occurs in yeast extract.

Despite the fact that inactivation of bacterial deaminase by exposure to molar phosphate buffer at pH 4 is not yet understood, is not always consistent, and doubtlessly is influenced by a variety of factors, these data afford confirmation that biotin somehow is concerned in activation of the system. Further study of the variables involved during inactivation and reactivation of the deaminase system is indicated before final conclusions can be drawn concerning the significance of biotin in these reactions.

Summary. Inactivation of bacterial aspartic acid deaminase activity by exposure of cells to pH 4 M phosphate buffer and its reactivation with biotin has been confirmed.

Received October 6, 1949. P.S.E.B.M., 1949, v72.

Binding of Phenolsulfonephthalein by Tissues and the Effect of Denaturation Thereon.* (17497)

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The present experiments were conducted to study (1) the binding of the organic anionic dye phenolsulfonephthalein (PSP) by homogenates of several tissues of the rat; (2) the effect of denaturation of tissue proteins on this binding; and (3) the competitive effect of native and denatured tissues on the binding of PSP by crystallized bovine plasma albumin. It has been previously established that certain proteins can form complexes with organic anions on both the acid and basic side of the isoelectric point. De Haan(1) and Marshall and Vickers(2) demonstrated that the anion PSP was bound appreciably by serum proteins and Grollman(3) showed that at the pH of normal blood the binding was primarily by serum albumin, the globulins binding very little dye. Klotz and Urquhart(4) showed that anions are also bound to β-lactoglobulin, hemocyanin and α_2 globulin, although to a much smaller extent than occurs with serum albumin. Ohta(5) working with tissue brei preparations presented evidence that both anionic and cationic dyes were adsorbed by tissue proteins. Denaturation of serum albumin by heat(6,7) or urea(7) abolishes the binding capacity for oleate and methyl orange anions. A different effect occurs with egg albumin in that heat denaturation increases the binding of alkylbenzenesulfonate detergents(8) and methyl orange.(7)

Experimental. Albino rats were exsanguinated by cardiac puncture or decapitation and weighed samples of the tissues were homogenized for approximately two minutes in M/15 phosphate buffer, pH 6.5. In the experiments, measured volumes containing 250-500 mg of the original tissue together with 1.0 ml of a solution of PSP in phosphate buffer pH 7 were placed in a cellophane bag 15 cm long and 1.5 cm in diameter. The samples were dialyzed at 5°C against M/15 phosphate buffer pH 6.5 in flasks which were slowly rotated for 40 hours to reach equilibrium; the total volume of the system was 100 ml. The bound PSP was calculated from the concentration of free PSP in the dialysate

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as determined colorimetrically by means of a photoelectric colorimeter with a 540 millimicron filter. The results were expressed in terms of the amount of dye bound by 400 mg of tissue, wet weight. In experiments with crystallized bovine plasma albumin (Armour) the protein was dissolved in M/15 phosphate buffer, pH 7.4, and used in a concentration of 5 g per 100 ml.

Tissues were denatured by the following methods (1) Heat: Tubes containing the tissue were placed in a bath of boiling water for 30 minutes; (2) Contact with urea in 6 Molar final concentration for three hours: The urea mixtures were placed in cellophane bags 30 cm in length and dialysed against distilled water for 5 days at 5°C to remove the denaturing agent. Aliquots containing 250 mg of tissue were used and the results were calculated on the basis of 400 mg of tissue. The effect of the tissues on the binding of PSP by albumin was determined by adding samples containing 400 mg of tissue to a mixture of 1.0 mg of PSP and 50 mg of albumin. The mixture was dialysed against M/15 phosphate buffer at pH 6.5. M/15 phosphate buffer was used for control of pH in all experiments and the pH of all solutions was determined with a glass electrode at the termination of each experiment. This buffer was found sufficient to maintain a constant pH during the course of the experiment. By mixing tissues with PSP and storing at 5°C for 48 hours with sampling at intervals it was found that the tissues employed did not cause a decrease in color of PSP.

TABLE I.
Binding of PSP by Native Tissues.

Tissue	No. of determinations	Range, units
Serum	6	60-104
Liver	10	30-87.5
Renal cortex	5	20.8-64
Renal medulla	5	29.4-60
Spleen	3	18.4-78
Testis	6	16.1-31.6
Pancreas	4	8-30

* The units are micrograms of dye bound by 400 mg of tissue or 0.4 ml of serum.

TABLE II.
Effect of Heat Denaturation on Binding of PSP by Serum and Tissues.

	Serum	Liver	Kidney	Pancreas
Unheated	62 (4)	65 (9)	44 (3)	30 (3)
Heated	8 (4)	125 (9)	75 (3)	55 (3)

Figures in brackets indicate the number of animals tested. Average values are given for micrograms of dye bound by 400 mg of tissue or 0.4 ml of serum.

Results and Discussion. The binding ability of liver, kidney, spleen, testis, pancreas and serum for PSP is shown in Table I. All of the tissues were found to bind the dye, although usually less than serum did; the liver was the most active tissue and pancreas the least. There was found to be some variation in the values obtained in the various experiments although the relative status of the tissues between themselves was maintained. The variation between the binding capacity of the liver of various rats was approximately ± 25%. No doubt this variation was due to the natural differences between the animals used. Denaturation of liver, kidney, and pancreas tissue proteins by heat or urea (Table II) resulted in a striking increase in the binding of PSP by all of these tissues. The increase in the binding by the liver tissue proteins denatured by both agents was of the same magnitude. In contrast to the tissues, denaturation of serum by heat markedly decreased its capacity for dye binding. The effect of denaturation of rat tissue proteins on dye binding is similar to the results obtained with egg albumin. These facts suggest that, in the denaturation process, either more foci of attachment for the PSP are opened in the binding proteins, as is the case with egg albumin, or that hitherto inactive proteins have become capable of binding.

When homogenates of native liver, kidney or pancreas were added to bovine albumin (Fig. 1) the binding of PSP was much less than the arithmetic sum of the dye bound by albumin alone plus the dye bound by the tissues alone. On the other hand no such decrease of binding was observed when heat-denatured tissue homogenates were added to bovine albumin; the mixture bound nearly as much PSP as the proteins did when tested separately. This experiment is interpreted as indicating that binding occurred between the

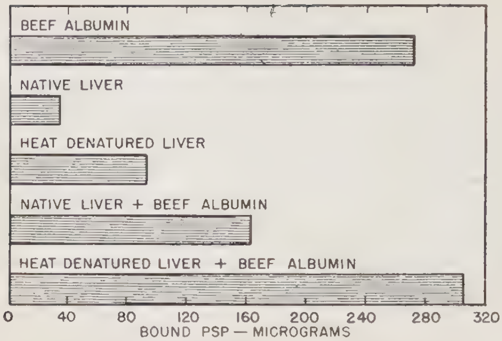

Fig. 1.

Effect of adding native, and heat denatured, homogenized tissue on the binding of phenolsulfonephthalein by crystallized bovine plasma albumin, 50 mg.

tissue proteins with the albumin molecule thus decreasing the available points of attachment for PSP in the system while heat denaturation of the tissue proteins decreased their attraction for albumin increasing the amount of dye anions bound by the mixture.

Summary. 1. Tissue proteins are capable of binding the anionic dye, phenolsulfonephthalein, in small amounts. All of the tissues examined bound less of the dye than did serum and different tissues appear to have different capabilities in this respect. Of the tissues tested liver bound most and pancreas least.

2. Denaturation of tissue proteins by heat or urea materially increased the ability of the tissue protein to bind the dye.

3. Native tissue proteins of the rat considerably decreased the binding of phenolsulfonephthalein by crystallized bovine albumin; this property was abolished by heat denaturation of the tissue proteins.

Received October 11, 1949. P.S.E.B.M., 1949, v72.

Effect of Aureomycin on the Herpes Simplex Virus in Embryonated Eggs.* (17498)

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Aureomycin was shown by Wong and Cox to have marked therapeutic activity against the viruses of the psittacosis-lymphogranuloma group.(1) They demonstrated that 49 of 55 treated chick embryos survived experimental infection with psittacosis through the 13th day, contrasted with survival of only 3 of 57 control embryos. These results were found to be essentially true for the other members of the psittacosis-lymphogranuloma group (lymphogranuloma venereum, psittacosis, SF strain human pneumonitis, mouse pneumonitis, feline pneumonitis, and meningopneumonitis F97 strain) as well as for the rickettsiae. In contrast with the marked activity *in vivo,* they conclude that the drug had no *in vitro* effect against these agents. Harned *et al.,* in a study on the acute toxicity of intravenously administered aureomycin hydrochloride for several laboratory animals, found the LD50 for the drug to be 134 mg/kg for mice and 118 mg/kg for rats.(2) Clinical accounts of successful treatment of

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* This work was supported by a grant from the U. S. Public Health Service.

The aureomycin used in this study was supplied by Lederle Laboratories Division of American Cyanamid Company, New York City.

Much of the preliminary work incident to this study was done by Dr. Lewis L. Coriell.

The activity levels for inactivated aureomycin were performed by Dr. Coleman Whitlock.

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diseases thought to be caused by herpes simplex such as Kaposi's Varicelliform Eruption,(3) dendritic ulcers of the cornea(4) and herpetic stomatitis(5) are being reported in increasing numbers. Because of the effectiveness of aureomycin against the larger viruses and the optimistic clinical reports against herpes simplex infections it seemed important to determine the *in vitro* effects of this antibiotic on the herpes simplex virus and to evaluate its *in vivo* action under the most favorable possible conditions in embryonated chick eggs.

Method and Materials. The toxicity of aureomycin was determined in chick embryos 10 to 12 days of age which averaged 50 g in weight. The eggs were prepared by the false air sac technique,(6) and the individual aureomycin dose dissolved in 0.2 ml of gelatin saline solution, was inoculated onto the "dropped" chorioallantois. The eggs were incubated at 96°F and 76% relative humidity. They were candled at least twice daily and death of the embryo was used as the end point. All dead eggs were cultured in Brewer thioglycollate medium for possible bacterial contamination. For drug controls penicillin in a concentration of 500 units/ml was used in some experiments and inactivated aureomycin in others. The aureomycin control was prepared by heating aureomycin at 56°C for 5 days, and was shown by bioassay to contain less than 0.1% of its original activity. Two lots of aureomycin were used. The first series of experiments was performed with batch A (Lederle lot No. unknown) and the later series with batch B (Lederle lot No. 7-9022B).

The virus used was egg adapted in this laboratory from the HF Rockefeller strain by 129 serial passages on the chorioallantoic membrane. The inoculum for each experiment was prepared from virus maintained in this laboratory by frequent passage. Different passages, each of which had a titer of approximately 10^9 infectious units per ml were used for each experiment. This strain of virus when properly diluted kills 95-100% of the chick embryos between the 69th and 90th hours. Embryos which died with virus plus drug were subcultured in other eggs to establish the presence of virus.

The *in vitro* effect was determined by mixing the drug and 10% suspensions of the infected membranes; incubating at room temperature for 4 hours; diluting the mixture sufficiently to obviate any *in vivo* effect; and injecting the inoculum onto the chorioallantoic membrane. Penicillin (500 units/ml) which had previously been shown to have no virucidal action was used as control.(7) To determine an *in vivo* effect one experiment was performed in which the virus and drug were inoculated into the yolk sac, the virus follow-

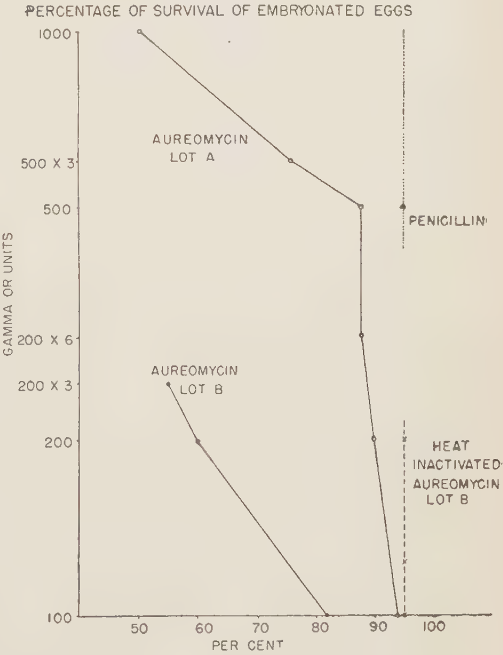

FIG. 1.

The toxicity of aureomycin and penicillin for embryonated eggs. The drug was inoculated onto the chorioallantoic membrane and death within 60 hours was taken as end point. Most points are the results from 24 or more eggs.

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ing the drug by 30 minutes. To detect minimal effects and insure maximal contact of drug with virus, additional experiments were done on the chorioallantoic membrane with the technique of inoculation as described in the toxicity studies. In one experiment the virus preceded the drug but in the others the drug was inoculated 30 minutes before the virus. In addition, in several experiments the drug was reinoculated at 12-hour intervals for a total of 3 or 6 doses.

Results. The toxicity of aureomycin for embryonated eggs is indicated in Fig. 1. The two batches of aureomycin, which were obtained at different times showed an appreciable difference in toxicity. Because of this, experiments to determine the effect of the larger doses on the virus could not be repeated with

TABLE I.

In vitro Effect of Aureomycin on Herpes Simplex Virus.

No. of infectious units inoculated into each egg	Conc. of aureomycin *in vitro* before dilution and inoculation	Amt. of aureomycin inoculated into each egg	Mortality of eggs inoculated with aureomycin-treated virus	Mortality of eggs inoculated with control (penicillin)-treated virus
5×10^5	4000 γ/ml	2 γ	12/12	12/12
5×10^4	4000 γ/ml	0.2 γ	10/10	12/12
5×10^3	4000 γ/ml	0.02 γ	12/12	12/12

TABLE II.

Mortality of Embryonated Eggs Inoculated on the Chorioallantois with Drugs and Then with Herpes Simplex Virus.

Approx. No. of infectious units inoculated	Aureomycin batch A						Aureomycin batch B (No. 7-9022 B)					
	100 γ/egg		200 γ/egg		500 γ × 3/egg		100 γ /egg		200 γ/egg		200 γ × 3/egg	
	Aureomycin	Control	Aureomycin	Control	Aureomycin	Control	Aureomycin	Control	Aureomycin	Control	Aureomycin	Control
5×10^5	20/21	22/22	17/23	27/27								
5×10^4	19/21	21/22	18/26	25/27	4/10	13/13	8/8	9/9	7/7	10/10	14/14	18/18
5×10^3	16/22	15/22	16/25	20/26	5/13	12/12	7/8	10/10	8/9	10/10	7/12	14/18
5×10^2					1/9	4/12	6/8	10/10	2/3	10/10	4/7	10/17
5×10^1							4/9	6/10	2/5	5/10		
5×10^0							3/9	2/10	1/10	2/10		

the second batch of aureomycin. Under the experimental conditions, aureomycin inactivated by heat, and penicillin were non-toxic. The LD50 of the least toxic lot of aureomycin for the chick embryo was 20 mg/kg. The *in vitro* effect of aureomycin on the herpes simplex virus is shown in Table I. The drug apparently has no *in vitro* action on the virus, under the conditions of the present experiments. The *in vivo* effect of aureomycin on the herpes simplex virus is shown in Table II. Because of the difference in toxicity and a possible difference in activity in the two lots of aureomycin, the results with each lot are recorded separately. There seems to be little difference in the effectiveness of the two lots of drug. Although in some of the dilutions small differences occurred between the controls and the drug treated embryos, at the critical dilutions with moderately large doses of drug and small amounts of virus there is no significant increased survival of aureomycin treated eggs. Analysis of the data also failed to show any significant delay in time of death of treated infected eggs.

Conclusions. (1) Aureomycin appears to be more toxic for the chick embryo than for most other laboratory animals. (2) There seemed to be a significant difference in toxicity between the 2 lots of aureomycin used. Incubation of aureomycin at 56°C for five days destroys the embryo-toxic principle as well as its antibiotic activity. (3) Aureomycin has no *in vitro* activity against herpes simplex virus. (4) As compared with its activity against the larger viruses, no similar *in vivo* effect against herpes simplex virus could be demonstrated.

Received October 17, 1949. P.S.E.B.M., 1949, v72.

Remission of Disseminated Lupus Erythematosus Induced by Adrenocorticotropin.* (17499)

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This report describes the effect of pituitary adrenocorticotropic hormone (ACTH) in inducing 3 temporary dramatic remissions in 2 patients with classic disseminated lupus erythematosus. There was a virtually complete clinical remission while the ACTH was being administered. A description of this phenomenon has not been previously published.

Case I: A.F., a 36-year-old white housewife was admitted complaining of fever, weakness, weight loss, and arthralgia of 9 months' duration. She appeared acutely ill, with a daily temperature of 101°F. There were erythematous, scaly plaques on both cheeks which also showed telangiectatic vessels and areas of atrophy. The eruption, in addition, involved the forehead, chin, ears, scalp, arms and legs. Scalp and pubic hair were sparse. There was a soft, blowing, systolic murmur over the 4th left intercostal space. The spleen was not palpable. Examination of the blood showed anemia, leukopenia, thrombocytopenia and an elevation of the sedimentation rate; protein and microscopic collections of red blood cells were constantly present in the urine. A section of the involved skin of the face showed changes usually seen in disseminated lupus erythematosus.

Beginning May 24, 1949, 150 mg of ACTH was injected intramuscularly in 6 divided doses daily for 5 days when they had to be stopped because further supplies of this scarce hormone were unavailable.

A remarkable change in the patient's condition was apparent within 12 hours after the injections were started. She appeared more alert, sat up in bed and expressed a desire for food for the first time in months. The arthralgia and myalgia disappeared. Within 24 hours, the temperature returned to normal and remained so as long as the hormone was given. The skin lesions began to fade and had almost disappeared by the 7th day after ACTH was started. The improvement in the appearance of the skin lesions continued for several days after the hormone was stopped, although the temperature was elevated on the day after ACTH was discontinued; thereafter the patient gradually relapsed into the clinical condition described before treatment was instituted. During the period of administration of ACTH, the eosinophils disappeared from the peripheral blood, the leukocyte count increased from 6,000 to 11,000, and the lymphocytes dropped from 48% to 23%. There was no appreciable change in the sedimentation rate or urinary findings. The urinary 17-ketosteroids increased from 5.2 mg to 27 mg. By June 11th the patient was irrational and appeared moribund. On the following day 150 mg of ACTH was again given daily and maintained for 3 days during which the skin lesions began to fade, she became rational, fever was partly controlled and appetite returned. On June 15th, when the supply of ACTH was exhausted, she again became irrational and died 4 days later.

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* The assistance and cooperation of Dr. Dale Doherty and Dr. S. Biloon in the clinical study of these cases is appreciated.

Case II: S.P., a 30-year-old white housewife was admitted complaining of swollen joints and an eruption on the face of 6 months duration. The eruption was made worse by exposure to sunlight. The classical erythematous lesions were present upon the face; warm, painful swellings existed over the wrists and metacarpophalangeal joints of both hands and there was a daily rise of temperature to 101°F. The spleen was moderately enlarged. Marked weakness of the upper and lower extremities was regarded by a neurological consultant as the result of peripheral neuritis. Examination of the blood showed anemia, leukopenia and an elevation of the sedimentation rate. There was only an occasional trace of protein in the urine from which porphyrin was also absent. The histopathologic picture of the erythematous skin was that usually seen in disseminated lupus erythematosus.

Beginning June 5, 1949, 100 mg of ACTH was injected intramuscularly in 4 divided doses daily for 5 days. Striking improvement followed and was manifested by disappearance of the swelling and pain in the wrists and joints of the hands, the fading of the skin lesions, diminution in size of the spleen, increase in muscular strength of the upper and lower extremities, return of temperature and appetite to normal; in addition, the eosinophils disappeared from the peripheral blood, the leukocyte count rose from 5,600 to 12,400 and the sedimentation rate returned to normal. Within 2 days after ACTH was discontinued the temperature began to rise, and 5 days later, there was a clinical relapse.

Discussion. After the first published demonstrations(1,2) that adrenal cortical activity induced either by administration of ACTH or the synthetic adrenal cortical steroid, 17-hydroxy-11-dehydrocorticosterone (Compound E of Kendall) was effective in alleviating acute nonbacterial arthritis, it appeared desirable to test these hormones in other members of the group of so-called "collagen diseases"(3) that also exhibit arthritis as part of the clinical picture. The effectiveness of Compound E in acute rheumatic fever has already been reported.(4) In addition, not only the arthritis, but cutaneous lesions of psoriasis disappear after the administration of ACTH(5) and return upon its discontinuance.

The temporary remissions induced by 5 days administration of ACTH represent an unequivocal, dramatic improvement in the course of a progressive, usually fatal disease. There is reason to expect that the continued administration of ACTH or Compound E would prolong the remission. It appears

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likely that the hormones only inhibit or mask the peripheral manifestations of rheumatoid arthritis, rheumatic fever, lupus erythematosus and psoriasis and do not affect the underlying causative disease mechanism, since a clinical relapse usually occurs promptly after cessation of hormone treatment.(2,4) In acute gouty arthritis, on the contrary, ACTH apparently completely aborts the acute attack.(1)

There are none of the usual signs or symptoms of adrenal insufficiency in the diseases benefitted by ACTH or Compound E and it is unlikely that the hormones act as substitution therapy for a deficiency. The expected change in the ketosteroids, eosinophils, lymphocytes, and total white count is evidence for adequate adrenal cortical function.(6) A "pharmacologic" extension of the usual physiologic properties of the hormones may be responsible for their action. In this connection, it is interesting to note the similarities between the effects produced by the salicylates and the adrenal hormone. Both groups of compounds lower fever and sedimentation rate, inhibit hyaluronidase,(7,8) cause hyperglycemia,(6,8) increase uric acid excretion(9,10) and are local anesthetics.(8,11) In addition, the salicylates and the adrenal hormones are both effective in rheumatoid arthritis and lupus erythematosus.(8)

Summary. Two patients with classical disseminated lupus erythematosus were given three separate courses of injections of adrenocorticotropic hormone (ACTH). In each case, there was temporary, striking clinical improvement and fading of the skin lesions, accompanied by the expected signs of increased adrenal cortical activity. These results suggest that continued administration of ACTH or Compound E may be of benefit in lupus erythematosus.

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Received October 17, 1949. P.S.E.B.M., 1949, v72.

A Test of Triazolopyrimidines on Mouse Sarcoma 180.* (17500)

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For nearly two years the major portion of a cancer chemotherapy program(1) has been devoted to a study of compounds that might serve as anti-metabolites to nucleic acid components. The studies were stimulated by the development of knowledge concerning the effects of anti-metabolites in bacteria and

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* This research was supported by funds from a grant of the American Cancer Society to the Memorial Hospital and from the Charles F. Kettering Foundation to the Wellcome Research Laboratories.

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animals(2-5) and the increasing information regarding details of nucleic acid metabolism.(6-8) Approximately 25 purines, 200 pyrimidines, 50 pteridines, 40 folic acid analogs and numerous miscellaneous heterocyclic compounds have been subjected to test. Among the compounds found to be most active in this program are those with anti-folic acid activity(9) and 2,6-diaminopurine(10) for the prolongation of survival of leukemic mice and some folic acid analogs for the inhibition of Sarcoma 180 and a few other tumors.(11-13) Compounds showing less inhibition of Sarcoma 180 in repeated tests have been found in each of the categories mentioned above. The reproducible, weak effects have been of interest in providing leads for study of related compounds.

A number of triazolo[*d*]pyrimidines (8-azapurines)(14) were among those which failed to show a satisfactory inhibitory effect in tolerated amounts against Sarcoma 180. The recent report(15) of the inhibitory action of 8-azaguanine (5-amino-7-hydroxy-l-v-triazolo [*d*]pyrimidine), also referred to as "guanazolo," on mouse mammary adenocarcinoma EO 771, on spontaneous mammary cancer in C3H mice, and on lymphoid leukemia in strain A mice redirected attention to our findings. The action of the guanine analog was interpreted by Kidder as indicating a similarity in guanine metabolism between *Tetrahymena gelii* and neoplastic tissues and, a "uniformity of various cancer tissues with respect to guanine metabolism".(15) An extension of our experiments with 8-azaguanine, 8-aza-adenine, 8-aza-isoguanine, 8-aza-2, 6-diaminopurine and 8-azaxanthine against Sarcoma 180 under conditions favoring demonstration of inhibitory action confirmed the ineffectiveness of these purine analogs at safe dosages. Inhibition was observed with each compound only when it was given in amounts sufficient to kill a large percent of the mice.

Materials and methods. Samples of the triazolopyrimidines were prepared independently by two of the authors, (L.F.C.)(16) and (G.H.H.). Dr. M. L. Crossley of the Calco laboratories kindly supplied two additional samples of 8-azaguanine. The technic used in the Sarcoma 180 test has been described.(11) In brief, small tumor implants (1-2 mm in any dimension) were made subcutaneously by trocar into the axillary region of CFW or RF mice, 18-22 g in weight. Twenty-four hours later the compounds suspended in gum acacia were injected intraperitoneally twice daily for one week. The dosages used were based initially on maximum tolerated doses determined by Dr. F. S. Philips, Pharmacology Section. Higher toxic doses were subsequently used when inhibition was not found at the safe levels. At the end of the injection period, the tumors were measured with calipers in two diameters. The degree of inhibition of tumor growth was graded as follows:

Marked inhibition (+), no growth of tumors in treated animals to a growth with an average diameter ¼ that of the untreated controls;

Slight inhibition (±), growth of tumors in treated animals from ¼ to ¾ the average diameter of the control tumors;

No effect (–), growth af tumors greater than ¾ the average diameter of the controls.

Discussion of results. The data are summarized in the table for only the highest levels tested with each compound. At lower toxic levels the compounds inhibited the tumors slightly and at non-toxic levels there was no effect. None of 8 samples of 8-azaguanine inhibited the development of Sarcoma 180, when tested at non-toxic levels. These levels,

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TABLE I.
Results with Triazolopyrimidines Tested Against Sarcoma 180.

SK No.	Name	Dose, mg/K/day	No. of mice[*]	No. of deaths	Tumor[†] inhibition
1054	5,7-diamino-l-v-triazolo (d)pyrimidine	250	20	9	— to ±
1059	5-hydroxy-7-amino-l-v-triazolo (d)pyrimidine	100	15	8	±
1149	5,7-dihydroxy-l-v-triazolo (d)pyrimidine	1500	15	8	— to ±
1150	5-amino-7-hydroxy-l-v-triazolo (d)pyrimidine	250	35	7	— to ±
1315	7-amino-l-v-triazolo (d)pyrimidine	125	35	23	— to ±

[*] A total of 410 treated animals were used in test groups of 5 mice each. Data for only the highest doses are tabulated. The remainder of the mice were tested at lower levels of the compounds as follows: SK 1054, 128-200 mg/K/day, 60 mice; SK 1059, 16-70 mg, 65 mice; SK 1149, 500-1000 mg, 55 mice; SK 1150, 64-200 mg, 85 mice; SK 1315, 63-100 mg, 25 mice. Two or more preparations of each compound were used.

[†] The grading of tumor inhibition is described in the section on Materials and Methods.

64-150 mg/K/day, were higher and initiated earlier (24 hours after tumor implantation) than in the experiments with adenocarcinoma EO 771 reported elsewhere. The preparations of 8-azaguanine varied in purity from one containing 40% of the guanine analog and 60% of the xanthine analog to several containing only one component as judged by a paper chromatogram.[†] The tumor inhibitory effects with the several samples of 8-azaguanine tested at 200 and 250 mg/K/day are considered insignificant because they were obtained at lethal levels. The analogs of isoguanine, 2,6-diaminopurine, adenine and xanthine also showed inconsistent inhibitory effects but only at lethal levels.

The failure of 8-azaguanine to inhibit selectively the growth of Sarcoma 180 is inconsistent with the concept that tumor cells in general possess a guanine metabolism comparable to that demonstrated for tetrahymena.(15) Sarcoma 180 would appear to be the first exception to the stated concept of "uniformity of various cancer tissues with respect to guanine metabolism".(15) The difficulties in generalizations concerning purine metabolism are further demonstrated by the observation(17) that with C 57 black mice bearing adenocarcinoma EO 771 there is an incorporation of guanine into nucleic acids of both normal and tumor tissue whereas in normal rats no significant incorporation has been observed.(6,7)

Regardless of the negative results obtained with 8-azaguanine against Sarcoma 180, it has been considered essential to test the compound in a spectrum of tumors. The results of these tests to be reported shortly(18) will reveal whether Sarcoma 180 possesses a metabolism unique among tumors with respect to guanine or merely represents a difference in response of Sarcoma 180 and mammary adenocarcinoma EO 771 to certain compounds such as found previously with folic acid analogs.(13)

Summary. 8-Azaguanine and 4 other triazolopyrimidines at tolerated doses were without inhibitory effect on Sarcoma 180.

[†] We are indebted to Dr. Aaron Bendich of the Protein Chemistry Division of Sloan-Kettering Institute for this analysis.

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Received October 17, 1949. P.S.E.B.M., 1949, v72.

Effect of Homologous and Heterologous Tissue on Mitosis in Heterozygous and Inbred Mice. (17501)

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(Introduced by Elton S. Cook)

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A significant inhibition of mitotic activity in the epithelial cells in the crypts of Lieberkühn in *dba* mice was produced by repeated subcutaneous injections of an aqueous alcohol-treated extract of beef spleen.(1) In the present experiments the effect of a single subcutaneous injection of splenic pulp of homologous and heterologous origin on mitotic activity of the cells in the crypts of Lieberkühn has been investigated in a highly inbred and a heterozygous strain of mice.

Experimental. Twelve *dba* line 1 mice from the Jackson Memorial Laboratories and 9 of the Rockland Farm all-purpose strain were employed in this study. The mice were approximately 6 to 8 weeks old and were allowed food and water *ad libitum*. Spleens were removed aseptically from donor mice of the same strains, pulped by passage through a fine meshed wire screen, and diluted 1:1 with Ringer's solution. A single subcutaneous injection of 0.5 cc was made into the groin of each experimental animal, and 7 days later the animals were killed, always at the same hour of the day in order to avoid introducing the diurnal variable in the mitotic cycle.(2) At autopsy a short segment of the small intestine located 20 mm distal to the pylorus was removed, fixed in Bouin's solution, dehydrated and embedded in paraffin. Histologic sections 8 mu in thickness were stained with hematoxylin.

Mitotic figures including those from late prophase to late anaphase were counted in the epithelium of the crypts of Lieberkühn in cross sections of the gut. Ten sections from progressive levels of the segment sufficiently separated to rule out counting the same figure more than once were used from each animal, and 50 fields in each of these sections were studied at a magnification of 970, a total of 10,500 fields being explored.

Two untreated groups of animals consisting of 5 Rockland Farm (Group I) and 5 *dba* line 1 (Group II) mice served as controls. The effect of spleen pulp from the Rockland Farm mice on mitosis in 4 Rockland Farm mice (Group III) and 5 *dba* line 1 mice (Group IV) was studied, and also the effect of *dba* spleen pulp on mitosis in *dba* gut epithelium (Group V). There were not sufficient *dba* animals to study the effect of *dba* spleen pulp on mitosis in Rockland Farm mice.

The significance of the data obtained from the different groups was determined by the formula(3) designed for small samples:

$$t = \frac{M_1 - M_2}{\sqrt{\left(\frac{N_1 \sigma_1^2 + N_2 \sigma_2^2}{N_1 + N_2 - 2}\right)\left(\frac{N_1 + N_2}{N_1 N_2}\right)}}$$

Levels of significance are expressed in percentage.

Results. No significant difference in mitotic activity in normal control mice of the highly inbred *dba* strain and the heterozygous Rockland Farm mice was found (Groups I and II, $t = 0.216$; significance level 20%). Homologous spleen in *dba* mice had no effect on the number of mitotic figures (Groups I and V, $t = 1.97$; significance level $> 20\%$) while homologous spleen in Rockland mice appeared to reduce the mitotic count slightly (Groups II and III, $t = 1.67$; significance level $< 5\%$). When splenic pulp from Rockland Farm mice was implanted in the *dba* mice, however, there was a marked increase in the number of mitotic figures observed (Groups I and IV, $t = 3.42$;

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TABLE I.
Effect of Homologous and Heterologous Spleen Pulp on Mitosis in Intestinal Epithelium.

Group	No. mice	Mitotic counts	
		Group avg ± S.D.	Range
I. Control *dba*	5	1.34 ± 0.250	(0.86–2.10)
II. Control Rockland	5	1.33 ± 0.267	(0.68–1.80)
III. Rockland spleen in Rockland	4	1.23 ± 0.295	(0.90–2.04)
IV. Rockland spleen in *dba*	5	1.55 ± 0.350	(0.82–2.46)
V. *dba* spleen in *dba*	2	1.21 ± 0.236	(0.80–1.74)

significance level 0.1%).

Discussion. The stimulation of mitotic activity obtained with heterologous tissue in these experiments tends to confirm the results of Breuhaus and McJunkin(4) using intraperitoneal injections of macerated rat kidney in normal and unilaterally nephrectomized rats.

Our results would indicate that a foreign tissue can influence mitotic activity as judged by the reaction of the epithelium in the crypts of Lieberkühn in the small intestine of the mouse, whereas a homologous tissue may not do so, as illustrated by the lack of significance of the data from *dba* mice receiving *dba* tissue. The borderline significance level for homologous tissue in Rockland mice, however, may possibly be attributed to the fact that this is a heterozygous strain and there would be a greater degree of difference between tissues in animals of this stock than between those in animals of the highly inbred *dba* line 1 strain.

Summary. The subcutaneous injection of spleen pulp from heterozygous Rockland mice into homozygous *dba* line 1 mice caused a significant increase in the mitotic activity in the epithelium of the crypts of Lieberkühn, whereas the injection of the spleen pulp from *dba* mice into mice of the same strain was without significant effect, and the injection of Rockland spleen into Rockland mice caused a mitotic decrease of borderline significance.

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Received October 20, 1949. P.S.E.B.M., 1949, v72.

Choline Deficiency in the Hamster. (17502)

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In a previous communication(1) it was noted that there is a correlation between the occurrence of fatty livers in choline deficient animals and the ability of the livers of normal animals of a given species to oxidize choline. Thus, rats,(2), mice,(3) and dogs(4) develop fatty livers on choline deficient diets and their livers possess an active choline oxidase.(5) In contrast, the guinea pig does not develop a fatty liver while living on a choline deficient diet(1) and the livers of this species are devoid of choline oxidase activity.(5) In the present work it has been found that the liver of the normal hamster shows only moderate choline oxidase activity and that liver fat accumulation in this species is less than that

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TABLE I.
Composition of Diets.

	1 %	2 %
Casein	10.0	6.0
Salts[7]	5.0	5.0
Lard	20.0	25.0
Cotton oil	10.0	5.0
Sucrose	38.0	13.5
Cod liver oil	1.0	1.0
Cystine	0.3	0.3
Cholesterol	0.5	0.5
Inositol	0.1	0.1
Glycocyamine	0.5	0.5
Peanut meal	—	30.0
Arginine	1.0	—
Liver ''L''*	3.0	3.0

* Wilson's Liver Fraction ''L'' obtained through the courtesy of Dr. C. E. Graham.

in rats on identical choline deficient rations.

Experimental. Liver Fat Studies. The animals were Syrian golden hamsters from a local colony of stock originally obtained from the General Biological Supply House, Chicago, Ill. They were housed in individual metal cages and offered the experimental rations *ad libitum* with water supplied at all times. The feeding technic was identical with that previously described for guinea pigs.(1) The basal diets are summarized in Table I. Each diet contained, in addition, per kilo of diet, thiamine 10 mg, riboflavin 10 mg, pyridoxine 10 mg, calcium pantothenate 50 mg, niacin 100 mg, *p*-aminobenzoic acid 50 mg, biotin 2 mg, folic acid 5 mg, α-tocopherol 200 mg, 2-methyl-l, 4-naphthoquinone 10 mg. The feeding experiments were continued for 35 days. At this time the animals were sacrificed by decapitation and liver samples analyzed for total lipids by a method previously described.(6) The results of these experiments are summarized in Table II.

The failure to obtain fatty livers in the hamsters on choline-deficient diet 1 is referable to the apparent inadequacy of this "synthetic" ration for this species. Food consumption was lower than in the animals on diet 2 and during the course of the experiment there was a net loss in weight. Fatty livers are not to be expected under these circumstances despite the choline deficiency.(7-9) No explanation is at hand for the modest fat accumulation observed when diet 1 was supplemented with choline. In contrast, all hamsters ingesting diet 2, which contained alcohol-extracted peanut meal were found to possess fatty livers and in the group shown in the table the lipid content varied from 11.9 to 19.1%. In another group of 14 hamsters fed the same diet, liver fat was found to vary from 9.4 to 28.1% with a mean of 15.3%. In general, there was a positive correlation between food consumption, growth rate and liver fat.

TABLE II.
Liver Lipids of Hamsters and Rats on Choline-Deficient Diets.

Group	Species	No. animals	Diet	Initial wt, g	Wt changes, g	Food intake, g/day	Liver lipids % wet wt
1	Hamster	6	1	96	—12	4.8	6.9
2	,,	6	1 + choline	93	— 7	5.2	9.4
3	Rat	6	1	100	23	6.1	13.9
4	,,	5	1 + choline	97	25	6.3	4.9
5	Hamster	7	2	101	8	6.9	15.5
6	,,	6	2 + choline*	102	7	6.7	7.5
7	,,	7	2 + methionine†	97	7	7.1	5.7
8	Rat	6	2	103	17	6.8	24.7
9	,,	5	2 + choline*				
10	,,	6	2 + methionine†	99	23	6.9	7.6

* Present as 0.6% of diet.
† Present as 0.8% of diet.

Choline Oxidase Studies. The animals were sacrificed by decapitation; liver and kidney

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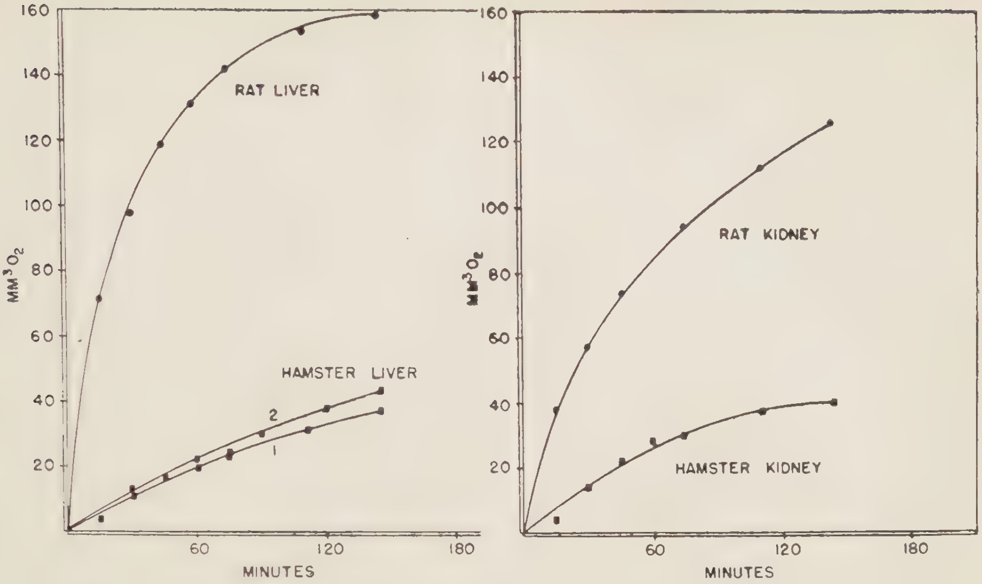

FIG. 1.

Choline oxidase activity of rat and hamster livers and kidneys. The substrate in each case was 1 mg of choline except for hamster liver where curves 1 and 2 represent the oxidation of 1 and 2 mg of choline, respectively. Theoretical oxygen uptake for 2 atoms of oxygen per choline molecule is 160 mm³.

were prepared by grinding in a mortar with sand and squeezing through muslin. Each Warburg vessel contained 0.5 ml of tissue suspension, diluted to 2 ml with M/20 phosphate buffer at pH 7.8. The choline chloride was dissolved in similar buffer and 1.0 mg of choline in 0.1 ml of buffer was added to the appropriate vessels at the expense of an equivalent volume of buffer. The vessels were then incubated at 37° in the conventional apparatus. The results are summarized in Fig. 1. In each case the control respiration in the absence of choline was subtracted before the data were plotted. It may be noteworthy that the basal respiration was of the same order of magnitude in rat and hamster. From the data shown in Fig. 1, it is apparent that hamster livers and kidneys do possess choline oxidase activity but this is markedly less than the activity of comparable rat tissues.

Because of the relatively poor growth and appetite of the hamsters on the peanut meal rations, as compared with the rats, it is not possible to determine whether the hamster is as susceptible to choline deficiency as the rat. It remains true, however, that, to date, the livers of all species which develop fatty livers on choline deficient rations have been found to possess choline oxidase activity in greater or lesser degree.

Summary. Young hamsters develop fatty livers when fed choline deficient rations containing peanut meal, although the mean increment in liver fat is only half that found in rats on the same diet. This may be correlated with the finding that hamster livers and kidneys possess markedly less choline oxidase activity than do the livers and kidneys of rats or may be due solely to the relatively poor appetite and growth of hamsters on these rations.

The authors' thanks are due to the Nutrition Foundation and to the Duke University Research Council for their support of this work; to Merck and Company, Rahway, N. J., for the crystalline B vitamins employed; to the Wilson Laboratories, Chicago, Ill., for the liver fraction "L," and to the Lederle Laboratories, Pearl River, N.Y., for the folic acid.

Received October 20, 1949. P.S.E.B.M., 1949, v72.

Cardiodepressive Effects of Thiomerin: Cardioprotective Attempts with BAL, Ascorbic Acid and Thiamin.* (17503)

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The new mercurial diuretic, thiomerin, (mercaptomerin, N.N.R.) has been studied intensively by several groups of investigators,(1-5) since its introduction by Lehman.(6) His original experiments with cats indicated deleterious intraventricular conduction effects, representing toxicity less than 1/160 that of mercurial diuretics in current use which do not contain the monothiol component. There is evidence that in this mercaptide derivative the toxicity has been reduced without impairing the diuretic efficiency of the mercurial compound,(3) whereas the dithiol—BAL reverses both the toxic and diuretic effects of mercurials.(7) We have previously shown that BAL, ascorbic acid, and thiamin, in that order of efficacy, prevent and partially reverse the deleterious conduction effect of mercuhydrin (meralluride, N.N.R.) and other organic mercurials in the isolated rabbit heart.(8) The purposes of this study were (1) to obtain comparative cardiotoxic and cardiolethal dosage levels of thiomerin and (2) to determine the cardioprotective effects of BAL, ascorbic acid and thiamin used before and after thiomerin in the isolated perfused rabbit heart.

Methods. The isolated rabbit hearts were perfused with a modified Locke solution at a constant temperature (37°C) and pH (7.4), and driven at definite varied rates with a thyratron stimulator, as in previous studies.(9) BAL, ascorbic acid, or thiamin, in predetermined amounts,(7) were injected into the tube leading to the aortic cannula either 10 minutes before or 10 minutes after thiomerin. Electrocardiograms corresponding to Lead I were obtained at various rates of stimulation, up to the production of A-V

TABLE I.
Doses of Thiomerin in mg/kg Heart Weight Producing Marked Conduction Delays (50% to 150% in QRS Width) and Rapid (Within 10 Minutes) Asystole in Isolated Perfused Rabbit Hearts.

	Conduction delays
8,100	mg/kg (thiomerin only)
8,300	’’ ’’ ’’
9,200	’’ ’’ ’’
11,000	’’ ’’ ’’
11,200	’’ ’’ ’’
13,500	’’ ’’ ’’
14,000	’’ ’’ ’’
14,600	’’ ’’ ’’
15,400	’’ ’’ ’’
15,800	’’ ’’ ’’
17,300	’’ ’’ ’’
17,700	’’ ’’ ’’
22,200	’’ ’’ ’’
	Rapid asystole
43,000	(after ascorbic acid)
48,000	(after thiamin)
54,000	(thiomerin only)
56,000	(after thiamin)
58,000	(after thiamin)
61,000	(thiomerin only)
62,000	(after ascorbic acid)
68,000	(thiomerin only)
69,300	(thiomerin only)
72,000	(after ascorbic acid)
77,700	(after thiamin)
88,700	(after ascorbic acid)
110,000	(after BAL)
128,000	’’ ’’
143,000	’’ ’’
157,000	’’ ’’

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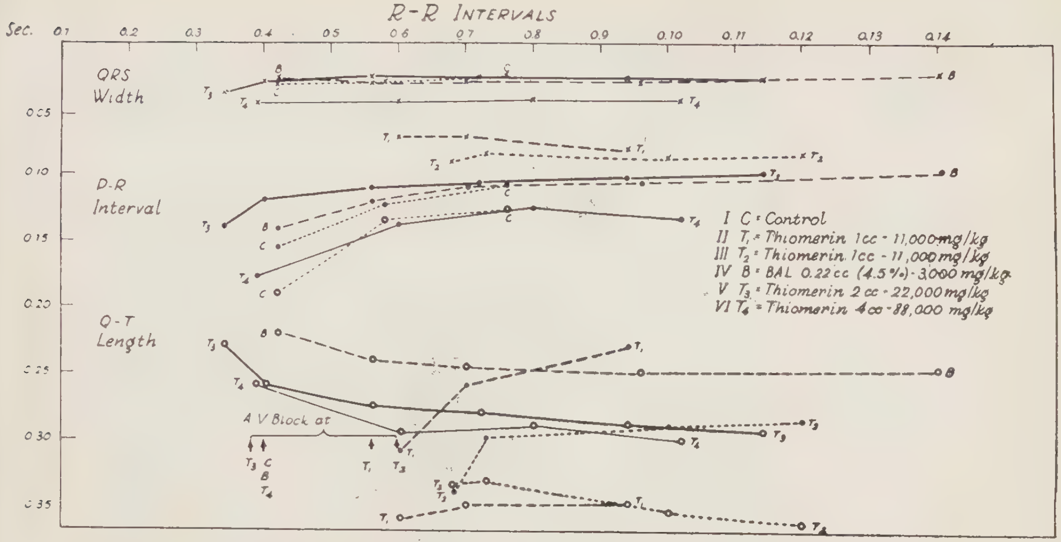

FIG. 1.

Example of usual reversal of thiomerin conduction effects by BAL, and BAL's cardioprotective value. Prolongations of P-R (dots), QRS (crosses), and Q-T (circles) intervals of the electrocardiogram at all rates of stimulation by thiomerin doses equivalent to 22,000 mg of mercury/kg—reversed by BAL. A total of 132,000 mg mercury equivalents per kg tolerated by the heart, with further deleterious but non-lethal conduction effects thereafter.

block, during the control and drug periods and analyzed by the magnifying Cambridge measuring instrument. P-R, QRS and Q-T intervals were then plotted against the R-R intervals for the control and other periods, as curves of recovery. Their prolongation or shortening at various rates of stimulation could thus be graphically evaluated. A total of 31 rabbit heart preparations were tested with thiomerin; 52 previous rabbit heart preparations provided comparative data for other mercurials.(7).

Results. 1. *Effects of thiomerin.* Thiomerin, like other mercurial compounds,(1,7) consistently and progressively prolonged conductivity in the ventricles, the atrioventricular conduction time, and the "electrical systole" or Q-T interval. Cardiac dilatation and slowing and lessened contractility resulted from large doses of thiomerin. It produced, to a much lesser extent than other mercurials,(7) monophasic type S-T segment displacements, doming and, rarely, reversals of the T waves. (Older batches of the drug were much more cardiotoxic, just as they showed greater irritation in the subcutaneous and intradermal tissues). Conduction effects were quite marked (prolongations of the QRS intervals of 50 to 150%) with doses of approximately 8,000 to 22,000 mg of mercury/kg of heart weight, with the new lots of the drug. (Older lots were similarly cardiotoxic in doses of 700 to 900 mg/kg). Rapidly (within 10 minutes) cardiolethal doses ranged from 54,000 to 69,000 mg of mercury/kg, with slow idioventricular rhythm and asystole occurring, to the exclusion of ventricular arrhythmias, as a terminal event. Transient ventricular ectopic beats and ventricular fibrillation occurred only once in 31 heart preparations after thiomerin and twice during control runs (rapid stimulation). The above figures indicate a comparative thiomerin cardiotoxicity of less than 1/200 that of mercuhydrin and 1/1,000 those of salyrgan-theophylline and mercuzanthin(7) in the isolated perfused rabbit heart. The cardiolethal ratios of thiomerin to mercuhydrin are less than 1 to 700, and of thiomerin to the other mercurials—1 to 1,800.

Thiomerin and BAL. The slight deleterious cardiac conduction effects of BAL in doses of 1800 to 3000 mg/kg have been previously dis-

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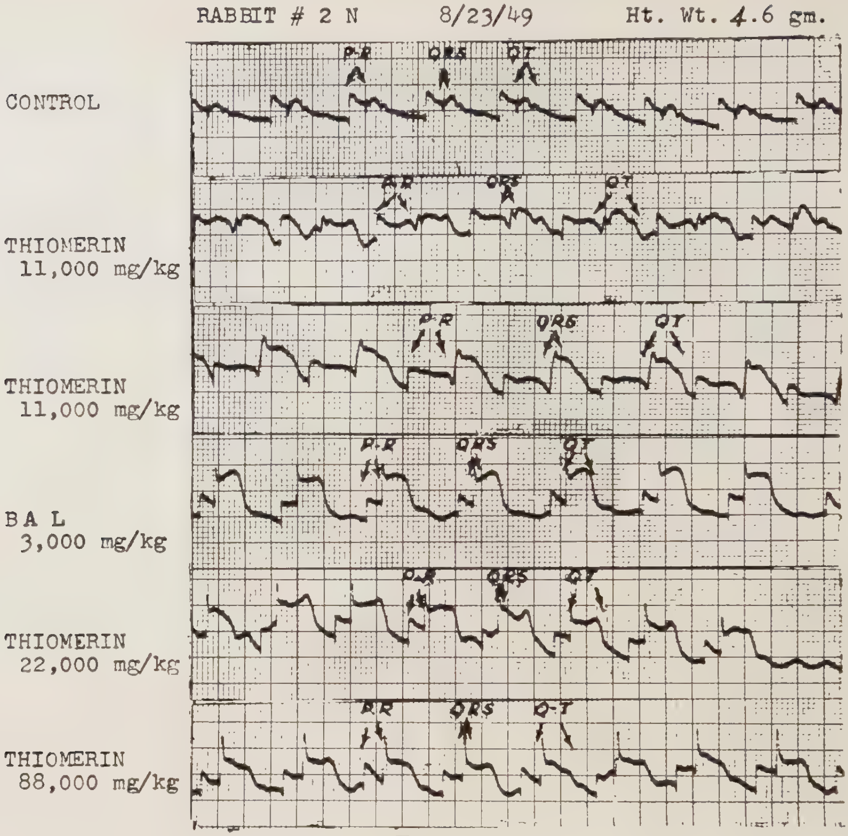

FIG. 2.

Electrocardiograms corresponding to lead I obtained at similar rates of stimulation during control, thiomerin, and BAL periods, 10 minutes apart, for same rabbit heart as in Fig. 1. The shortening of the P-R, QRS, and Q-T intervals by BAL, and their lengthening by thiomerin, much less after BAL than after control runs, are clearly seen.

cussed by us.(7) Thiomerin was tolerated thereafter in doses up to 157,000 mg of mercury/kg of heart weight (Table I), or approximately 2 times the usual cardiolethal dose, when preceded by BAL. BAL, likewise, markedly reversed the prolongation of the electrocardiographic intervals by previous injections of thiomerin into the closed perfusion system (Fig. 1 and 2).

Thiomerin and ascorbic acid. Ascorbic acid in doses of 4000 to 6000 mg/kg of heart weight caused slight deformations of the electrocardiographic patterns as previously reported.(7) In contrast to its action in increasing the cardiolethal dosages of mercuhydrin and the more toxic mercurials,(7) it had no such effect in respect to thiomerin (Table I). However, the slight conduction effects of small doses of thiomerin were often partly reversed by ascorbic acid in the above dosage (Fig. 3).

Thiomerin and thiamin. Thiamin, in doses of 500 to 1000 mg/kg of heart weight caused slight changes in the electrocardiographic patterns as noted previously.(7) Again, in contrast to its effect on mercuhydrin, it failed to protect against more than the usual cardiolethal doses of thiomerin (Table I). Reversal of deleterious conduction changes resulting from small doses of thiomerin was the exception rather than the rule for thiamin (Fig. 4).

Comment. Samples of thiomerin received in the first half of the year 1949 showed cardiodepressive effects corresponding to doses 20 to 25 times those of mercuhydrin (700 to 900 mg/kg vs. 20 to 36 mg/kg respectively). Newer lots of the drug, however, showed a

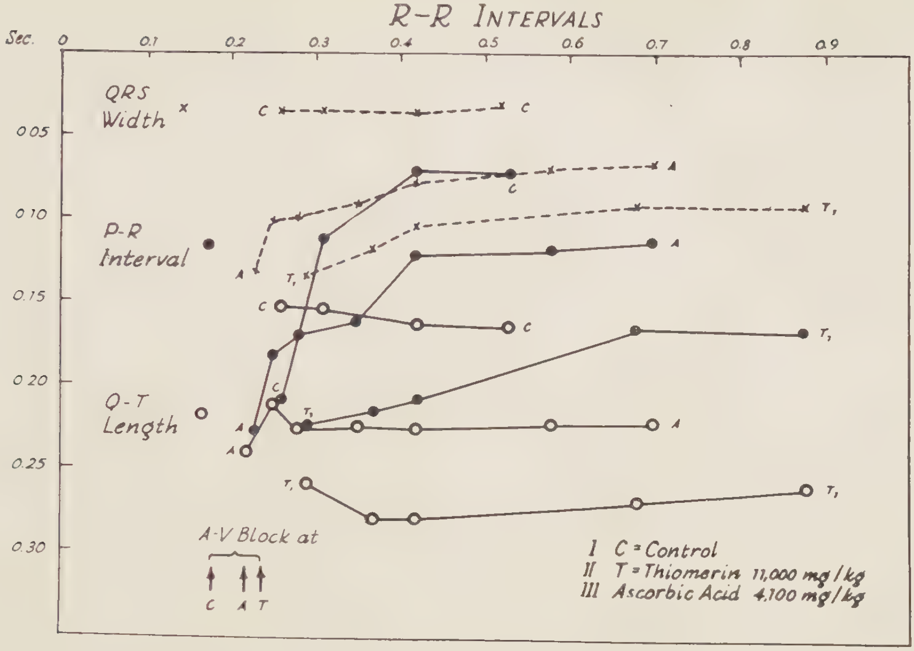

FIG. 3.

Example of frequent reversal of small dose thiomerin conduction effects by ascorbic acid. Prolongations of P-R, QRS and Q-T intervals of the electrocardiogram at all rates of stimulation by thiomerin are reversed, though less markedly than by BAL (Fig. 1), in all instances, by the large dose of ascorbic acid.

cardiac toxicity of less than 1/200 that of mercuhydrin. This ratio corresponds to Lehman's original figure of less than 1/160, measuring the comparative effects of the two drugs in prolonging the QRS interval in cats. Such marked innocuousness of thiomerin from the cardiotoxic standpoint is also emphasized by the absence of ventricular ectopic rhythms in the rabbit heart preparations, or in human subjects(3) given the drug subcutaneously and intravenously. Corresponding absence of marked subcutaneous and intracutaneous irritation both in animal and human subjects(10) of the newer lots of the drug is in marked contrast to the tender subcutaneous nodules and sterile abscesses, and erythematous, papular, vesicular and even necrotic intracutaneous reactions of other mercurials. It is of interest that whereas BAL, ascorbic acid and thiamin raised the maximum cardiolethal (causing asystole within 10 minutes) doses of meralluride to above 10, 7.6 and 2.6 times the M.L.D. of the mercurial alone, respectively,(7) only BAL was successful in increasing the thiomerin tolerance, and that only to the extent of doubling the cardiolethal dosage. We can only speculate as to what the mercaptan linkage has to do with rendering thiomerin less toxic to the cardiac enzyme systems, and BAL of relatively little further cardioprotective effect. Work in progress in our neurochemistry laboratory indicates that oxidation is markedly suppressed or abolished for at least 30 minutes in rat heart slices by mercuhydrin.(11) The depression of respiration of yeast brought about by phenylmercuric nitrate is prevented by cysteine and homocysteine,(12) but more direct biochemical evidence relative to the cardioprotective effects of sulfhydryl compounds is not available. Likewise, the failure of ascorbic acid to in-

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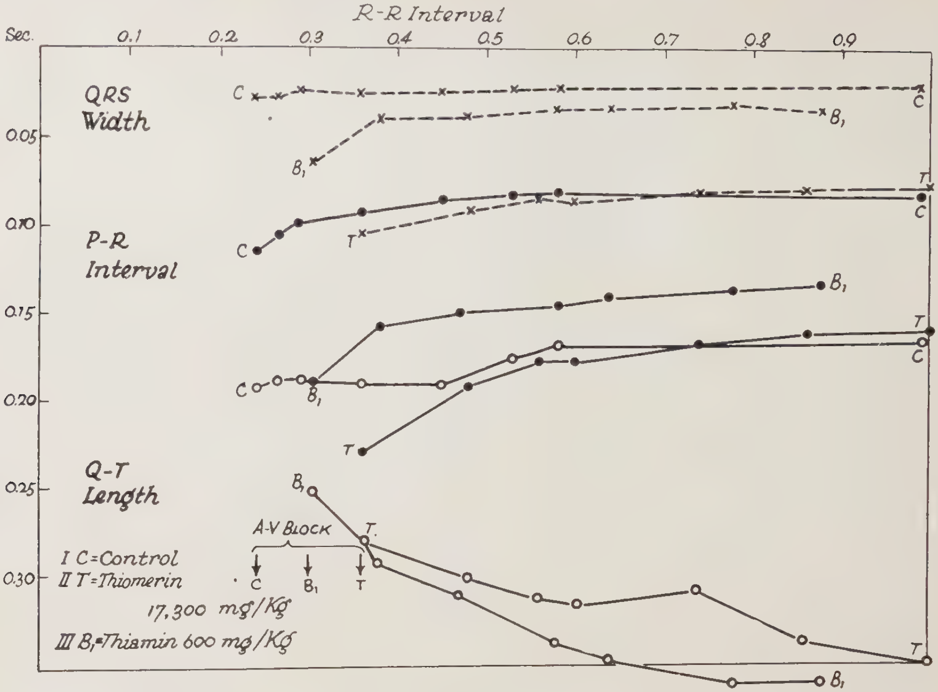

FIG. 4.
Example of infrequent reversal of small dose thiomerin conduction effects by thiamin. Prolongations of P-R and QRS intervals of the electrocardiogram at all rates of stimulation by a moderate dose of thiomerin are reversed by thiamin, as recorded for the three periods 10 minutes apart. The markedly prolonged Q-T intervals resulting from thiomerin are not shortened by thiamin. Compare similar results for mercuhydrin.[7]

crease further the cardiac tolerance of a mercurial compound with a mercaptide linkage may or may not be connected with possible thiomerin potentiation of the already present ascorbic acid in the cytochrome oxidase system in its active reductant state. This cannot be substantiated without further biochemical studies. For what obscure reason thiamin, and presumably its phosphorylated enzyme product — cocarboxylase, increases, though slightly, the cardiac tolerance to organic mercurial compounds other than thiomerin cannot be answered in the present state of our knowledge.

Summary and conclusions. 1. In the isolated rabbit heart thiomerin appeared to slow atrio-ventricular and intraventricular conduction and prolong the "electrical systole" (Q-T interval) in doses, as to mercury content, over 200 times those of meralluride, and 1000 or more times those of other commonly used mercurial diuretics. The rapidly cardiolethal dosages bore even larger ratios. Idioventricular slowing and asystole, associated with marked cardiac dilatation and failure of contractility, and not ventricular ectopic rhythms, constituted the lethal manifestations of the thiomerin perfused heart.

2. In contrast to the marked increase in cardiac tolerance to other organic mercurial compounds as a result of preliminary administration of BAL, and to a lesser extent, of ascorbic acid and thiamin, BAL approximately doubled the cardiolethal dosage of thiomerin and the two vitamins were not significantly cardioprotective against the monothiol mercurial.

3. BAL markedly reversed the deleterious conduction effects of even large doses of thiomerin; ascorbic acid did so frequently, after relatively small doses; thiamin reversed the milder effects of thiomerin but in a minority of cases.

Received July 20, 1949. P.S.E.B.M., 1949, v72.

Cardiodepressive Effects of Mercurial Diuretics. Cardioprotective Value of BAL, Ascorbic Acid and Thiamin.* (17504)

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The use of mercurial diuretics is attended by cardiac abnormalities. The more serious, and even fatal, immediate reactions are ventricular fibrillation, preceded by ventricular tachycardia and ectopic beats, or, infrequently, cardiac asystole.(1) Elevation and other deformations of the S-T and T segments, notching of the QRS, prolongation of the P-R and QRS intervals up to alternating bundle branch block, auricular fibrillation and flutter, also occur, in human cases and in animal experiments.(1-3) BAL is conceded to be the most effective dithiol compound in combatting the toxicity of mercurial compounds, as well as neutralizing their diuretic effects.(4) Ascorbic acid likewise reputedly has antitoxic as well as synergistic diuretic effects when administered with mercurials.(1) Thiamin, reported beneficial in arsenical poisoning,(5) has not been used to counteract mercurial toxicity. Neither thiamin nor ascorbic acid have known direct cardiac effects, except that the T wave may become inverted after thiamin.(6) BAL has been observed to cause, beyond slowed cardiac rates, elevated S-T segments and deep S waves.(7)

The purposes of this study were to obtain specific conductive and electrical systole effects of, particularly, mercuhydrin in the isolated rabbit heart; to compare its myocardial toxicity with that of other organic mercurials; and to evaluate the detoxifying effects of BAL, ascorbic acid and thiamin used before and after the mercurial compounds.

Methods. As in our previous investigations(8,9) with the isolated rabbit hearts, they were perfused per aorta and coronary arteries with a solution kept at 37°C, pH 7.4, and oxygenated with 5% CO_2 in oxygen. Electrocardiograms corresponding to lead I were obtained, as the auricles, or ventricles with the auricles cut off, were driven at various rates by means of a thyratron-type stimulator. The P-R, QRS and Q-T intervals were measured at various rates of stimulation with a Cambridge measuring instrument and graphed against the R-R intervals as curves of recovery (relative refractoriness). Control tracings were followed by the injections of the drugs in appropriate dosage, mercurials followed by the supposed antagonists, or vice versa. Ten minute intervals between doses of the same or different drugs allowed records of the maximum effects, and, together with rest periods between salvos of stimuli, elminated the noxae of fatigue. The pH of the perfusate was not altered more than 0.5 unit by any of the drugs added, so that the acids (thiamin hydrochloride, ascorbic acid) administered needed no buffering. Moreover, our own studies(11) indicate that a pH range of 6.5-8.0 of the perfusate is unaccompanied by marked changes of the electrocardiographic intervals measured. A total of 52 experiments, 6 dealing with salyrgan-theo-

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* With the technical assistance of James E. Amburn. Supporter in part by a grant from Campbell Products, Inc., New York.

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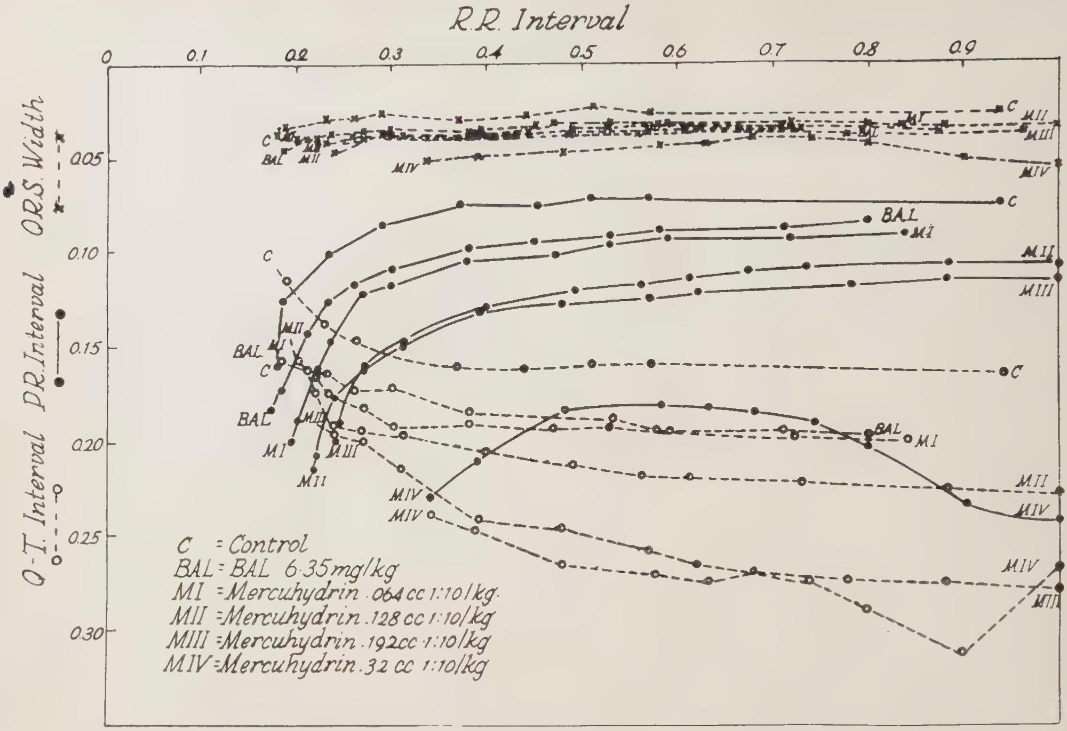

FIG. 1.

BAL (0.22 cc saturated aqueous soln.) prolongs A-V and I-V conduction times and Q-T intervals at all rates of stimulation (R-R intervals). Despite these deleterious effects, the total mercuhydrin tolerated thereafter amounts to 780 mg of mercury/kg of heart weight, or more than 10 times the usual cardiolethal dose of the mercurial. Times—sec.

phylline, 6 with mercuzanthin, and 40 with mercuhydrin, constitute the basis of the following data. The rabbits weighed 1.5 to 2 kg and the hearts 4 to 6 g. The perfusate volume was 1 liter. Hence, to translate dosages of mg/kg of heart weight into concentrations of mg % of perfusate, one need only divide by the factor 20 (*i.e.* $\frac{1000}{5 \times 10}$). Dosages on figures per kg of body weight must be multiplied by the factor 200 to get approximate values per kg of heart weight.

Results 1. *Effects of Mercurials.* Salyrgan-theophylline and mercuzanthin were usually rapidly (within 10 minutes) fatal to the rabbit hearts at doses of 30 mg of mercury/kg of heart weight, or above. Block of severe degree, A-V nodal or intraventricular (QRS widening of 50 to 150%), generally occurred at a dosage of 7.5 mg of mercury/kg or above. That mercuhydrin was less toxic, as noted by previous investigators,(10) was emphasized by its cardiolethal dosage of 75 mg of mercury/kg or over, and the occurence of marked impairment of conduction following doses of 36 mg of mercury/kg or over (Fig. 2, 4, and 6; Table I).

2. *BAL and Mercuhydrin. BAL* in saturated aqueous solution (4.5%), administered in a dosage of 1800 mg/kg of heart weight caused some impairment of conduction and lengthened the Q-T interval. The dosage of mercuhydrin tolerated thereafter, however, increased up to 780 mg of mercury/kg without immediately killing the heart, although further deleterious changes in conduction and refractory period followed (Fig. 1). When conduction impairing doses of mercuhydrin (*e.g.* 36 mg of mercury/kg) were followed by BAL 1800 mg/kg), there resulted a marked re-

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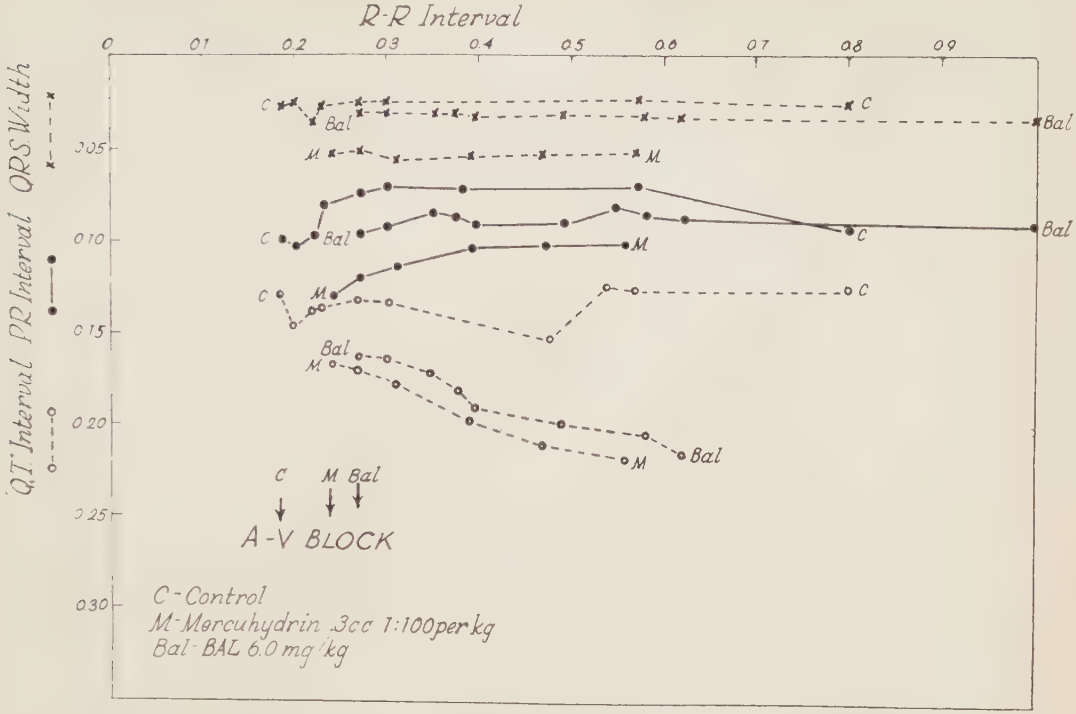

Fig. 2.

The prolongation of QRS, P-R, and Q-T intervals at all rates of stimulation by a blocking dose of mercuhydrin (36 mg of mercury/kg heart weight) is reversed by BAL in that order of decreasing efficacy.

versal of A-V and intraventricular block, though the Q-T interval and the absolute refractory period were further lengthened (Fig. 2). In one instance of complete cardiac asystole after mercuhydrin, BAL caused resumption of idioventricular rhythm, though the heart failed to react to electrical stimulation. S-T changes of monophasic current type and doming or reversals of the T wave frequently followed the exhibition of either mercurials or BAL.

3. *Ascorbic acid and mercuhydrin.* Ascorbic acid in doses corresponding to therapeutic ones in man did not affect the electrocardiogram. Doses of 4500 to 5400 mg/kg at times very slightly impaired conductivity and prolonged the Q-T interval, with minimal S-T and T changes. The maximum tolerated amount of mercuhydrin administered after such doses of ascorbic acid was 570 mg of mercury/kg, although usually only much lower doses could be protected against (Fig. 3, Table I). Ascorbic acid used after blocking doses of mercuhydrin (36 mg of mercury/kg) caused, in general, partial reversal of A-V and possibly I-V block, and not infrequently further slight prolongation of the Q-T interval and the absolute refractory period of the recovery curves of conductivity (Fig. 4). The detoxifying effects were intermediate between those of BAL (supra) and thiamin (infra).

4. *Thiamin and mercuhydrin.* Thiamin in doses corresponding to therapeutic ones in man did not affect the electrocardiogram. Doses of thiamin of 570 to 780 mg/kg of heart weight at times impaired conductivity and prolonged the Q-T interval, with slight S-T and T changes. Mercuhydrin administered after such amounts of thiamin was tolerated up to 196 mg/kg without killing the heart, although increasing block developed (Fig. 5). In one instance a mercuzanthin dose of 105 mg of mercury/kg similarly (Table I) produced A-V dissociation without immediate death of the heart after an apparently protective dose of thiamin (600 mg/kg). Thia-

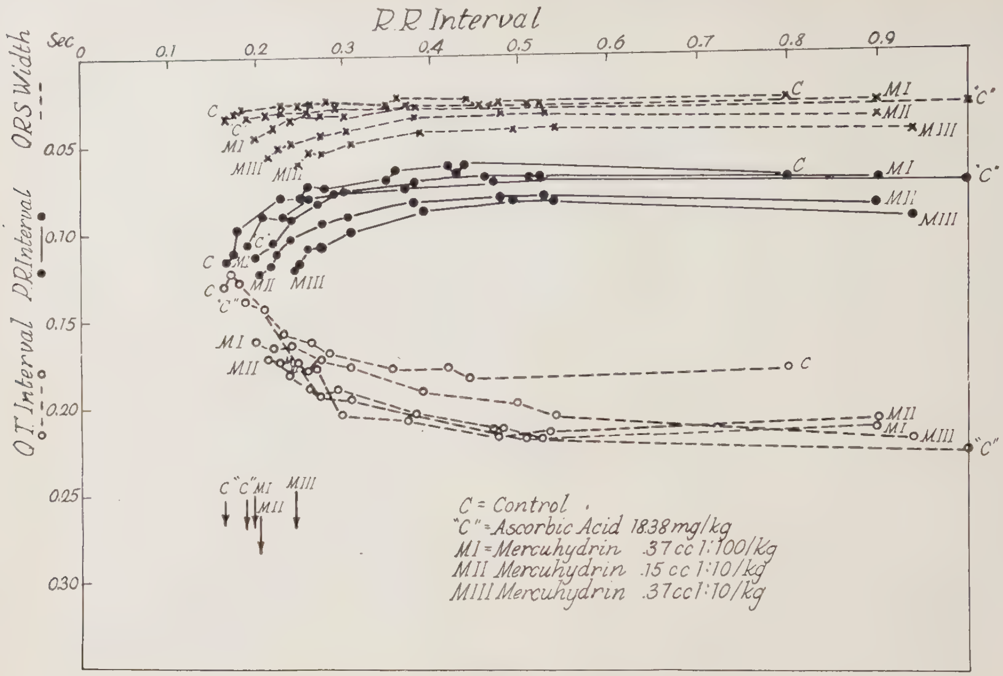

FIG. 3.

Ascorbic acid has practically no effect on this heart preparation except for prolonging the Q-T interval and slightly decreasing the blocking stimulus rate (see arrows). 570 mg of mercury/kg of body weight, or more than 7 times the usual cardiolethal dose, is tolerated thereafter with few further deleterious effects on the measured intervals.

min administered in above doses after blocking doses of mercuhydrin caused partial reversal of the prolonged P-R and QRS intervals, while prolonging the Q-T intervals further (Fig. 6). This action was similar to but less marked than that of BAL and ascorbic acid under similar conditions (supra).

5. *Other drugs and mercurials.* Theophylline ethylene diamine in doses of 3600 mg/kg apparently increased the tolerance of the isolated rabbit heart to mercuhydrin (*e.g.* 120 mg of mercury/kg with non-lethal A-V dissociation, Table I), while itself causing some block and particularly prolongation of the electrical systole.(11) The protective effects of theophylline have been previously reported,(2) associated, perhaps, with the increased absorptive and diuretic results of its combinations with mercurials,(2,12) as well as coronary dilation since pitressin had the opposite effect (Table I). Epinephrine, 6 mg/kg, partially reversed the effects of blocking doses (36 mg of mercury/kg) of mercurials on the P-R, QRS and Q-T intervals, without causing ventricular fibrillation.(11) DeGraff and Lehman(2) had previously reported "improvement" in hearts poisoned by mercurials following ephedrine. Since magnesium sulphate has been reported to have detoxifying value against mercurial diuretics,(13) doses up to 45 to 120 g/kg were employed before and after mercuhydrin with failure to demonstrate any protective or beneficial effects. Marked A-V (not I-V) block and prolongation of the Q-T interval were produced following $MgSO_4$ both before and after mercuhydrin, and the cardiolethal dose of the latter was still 75 to 125 mg of mercury/kg after $MgSO_4$. Niacin likewise failed to increase the tolerance of the isolated rabbit heart to mercurials, and in doses much larger than the corresponding therapeutic ones in man, *e.g.* up to 9000 mg/kg, caused slight impairment of conduction (A-V, rather than I-V), and prolongation of

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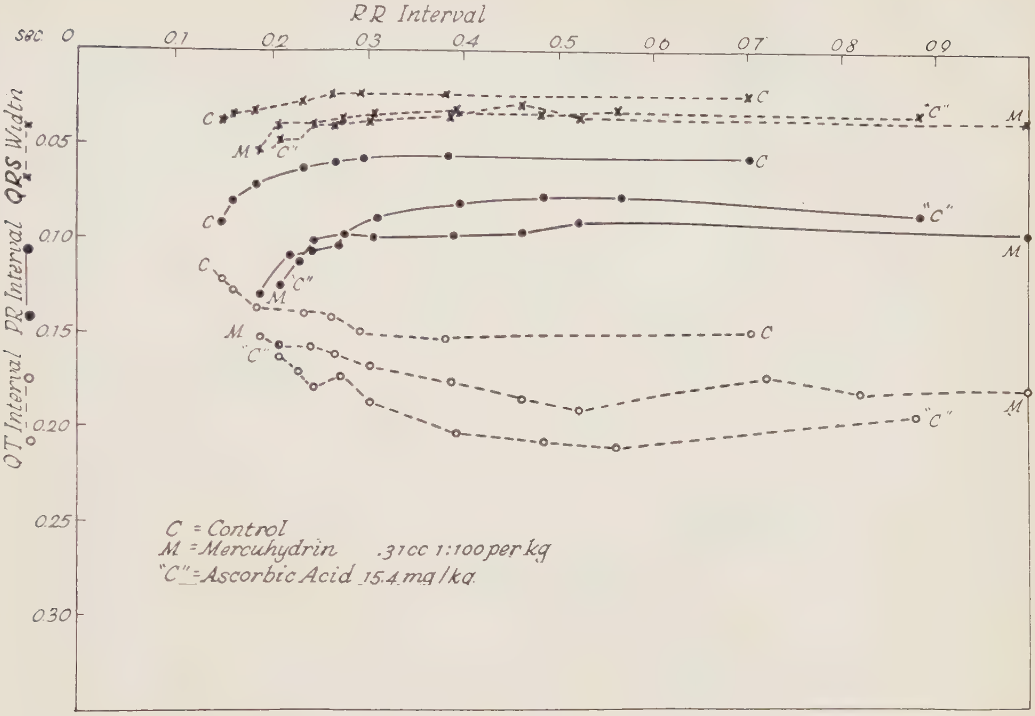

FIG. 4.

The usual effects of a blocking dose of mercuhydrin (36 mg of mercury/kg of heart weight) are reversed as regards A-V and I-V conduction only slightly, and at lower rates of stimulation, by ascorbic acid in this preparation; the electrical systole is further prolonged.

the Q-T interval and the absolute refractory period of conductivity.(11)

Comment. Since the lethal effects of intravenous mercurial diuretics result primarily from cardiac toxicity,(2) it is pertinent to compare the doses of mercurials fatal for the isolated rabbit hearts with the known M.L.D.'s of these drugs in intact animals. In terms of mercury content, mercuhydrin was fatal in dogs in a dose of 45-61 mg/kg of body weight,(1) and salyrgan-theophylline in cats at 35 mg/kg.(2) Our corresponding figures for the two drugs are 75 mg/kg and 30 mg/kg of heart weight respectively. These doses of mercurials produce rapid cessation of cardiac activity as compared with the slower cumulative effects of the M.L.D.'s as defined for intact animals. Although our results are not quantitative, it appears that BAL, ascorbic acid and thiamin offered, in that order, significant protection to the heart against mercurial toxic and lethal effects. *The maximum tolerated (sublethal) doses of mercuhydrin following BAL, ascorbic acid, and thiamin, were 10.0, 7.6, and 2.6 times the previously noted M.L.D. of the mercurial alone in our preparation of the perfused isolated rabbit heart, respectively.* Each of them, likewise, showed definite reversal of mercury-induced conduction defects and, usually, electrical systole prolongation.

Further studies indicate that mercaptomerin (the disodium salt of N (γ-carboxymethylmercaptomercuri-β-methoxy)propyl camphoramic acid, thiomerin-Campbell Products) is less than 1/200 as cardiotoxic as mercuhydrin. Slight cardioprotective effects also result from the exhibition, before or after thiomerin, of BAL, but not of thiamin or ascorbic acid.(14) The mechanism of BAL detoxication of mercury has been adequately discussed elsewhere.(4) We can offer no explanation of the actions of thiamin and ascorbic acid, other than possible

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14. Ruskin, A., and Johnson, J. E., to be published.

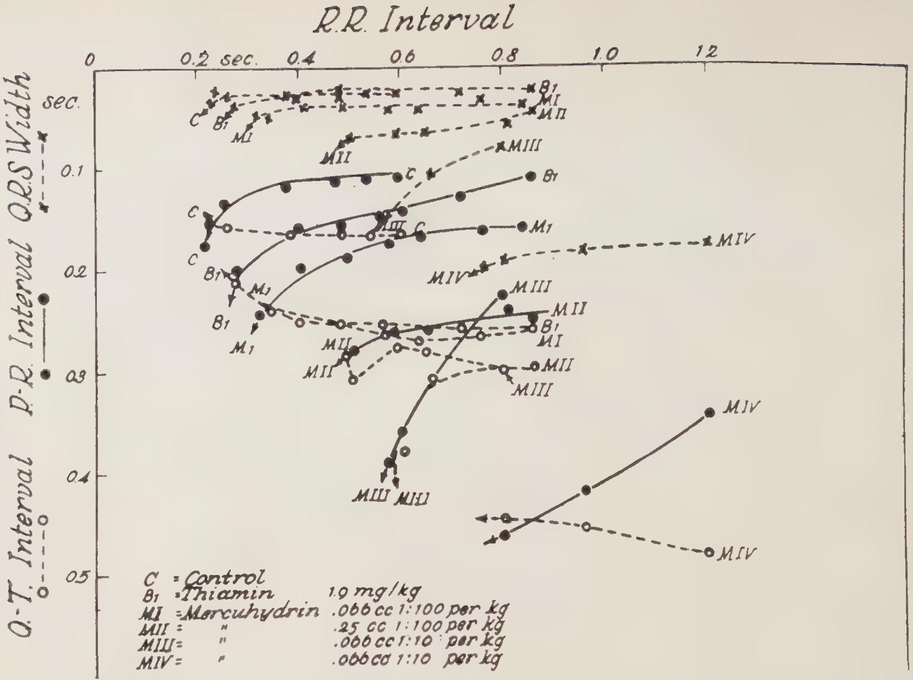

Fig. 5.

The large dose of thiamin has increased A-V conduction time and the Q-T intervals at all rates of stimulation in this heart. Nevertheless, mercuhydrin is tolerated, thereafter, though with markedly deleterious effects, up to 195 mg of mercury/kg of heart weight, or almost 2 times the usual cardiolethal dose of mercuhydrin alone.

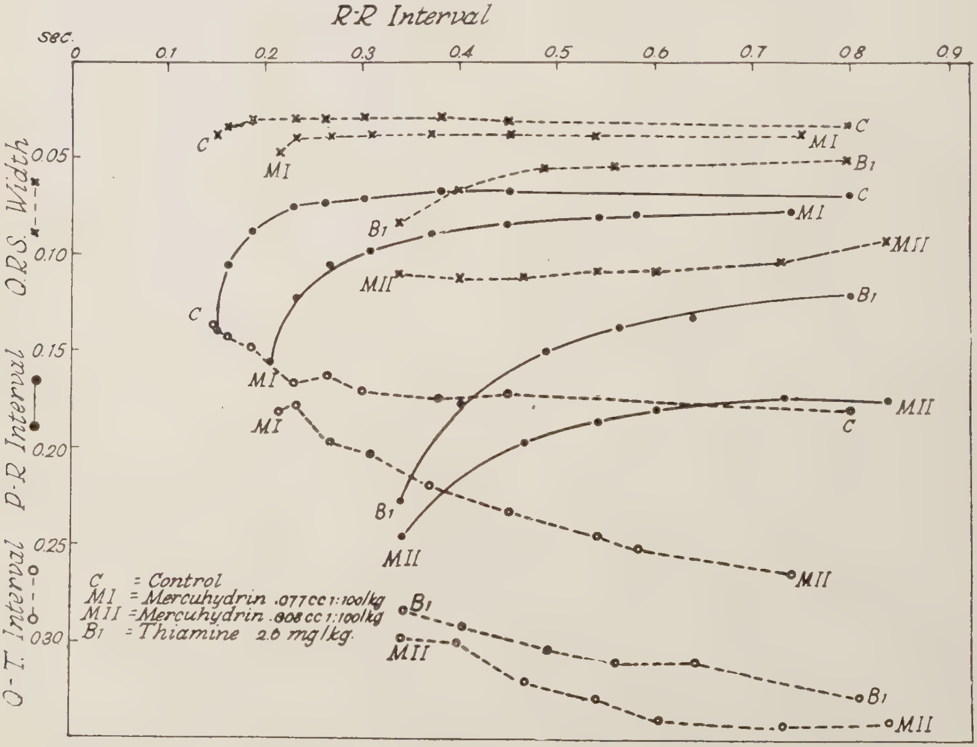

Fig. 6.

A total of 45 mg of mercury/kg of body weight administered in two successive doses of mercuhydrin has caused marked prolongation of A-V and I-V conduction and electrical systole in this preparation. Thiamin then effected marked shortening of conduction times and a slight shortening of Q-T intervals without changing the blocking stimulus rate.

TABLE I.
Representative Doses of Mercuhydrin and Other Mercurials in mg/kg Heart Weight Producing Marked Conduction Delays (50% to 150% in QRS Width at 150 Beats/min.) and Rapid (Within 10 Minutes) Asystole in Isolated Perfused Rabbit Hearts.

Conduction delays mg/kg		Rapid asystole mg/kg
	Salyrgan-theophylline	
7.9		29.6
10.8		36.2
11.8		44.1
	Mercuzanthin	
7.4		30.8
12.6		46.7
44.2 (after thiamin)		105.4 (after thiamin)
	Mercuhydrin	
17.8 (after pitressin)		75.8
29.8		88.2
30.4		95.6
38.8		112.7
40.0		116.2 (after $MgSO_4$)
40.7		118.9
42.1		124.6
44.1		144.4
48.6		148.5
48.8		149.4
53.9		169.4 (after thiamin)
56.1		195.2 (” ”)
62.3		356.7 (after ascorbic acid)
66.8		418.8 (” ” ”)
120.4 (after aminophylline)		570.6 (” ” ”)
242.6 (after BAL)		696.6 (” BAL)
		780.5 (” ”)

potentiation of the cellular enzyme systems incompletely destroyed by mercury. Biochemical studies, utilizing the Warburg technic, now being conducted in our neuro-chemistry laboratory, may substantiate this hypothesis.(15) Other substances concerned with cellular oxidation, such as niacin and riboflavin, are without effect on mercurial toxicity.(11) The favorable effects of theophylline ethylene diamine and epinephrine confirm previous similar observations. Magnesium salts, while apparently stimulating the contractility of the preparation,(11) produce, with mercuhydrin, additional deleterious conduction effects, without changing the cardiolethal dosage of the mercurial compound.

Summary. Mercuhydrin uniformly caused conduction delays in the A-V node and ventricles of the isolated rabbit heart. Electrical systole (Q-T interval) was likewise prolonged, and failure of A-V conduction (dropped beats) produced at lower than usual rates of stimulation—all at doses above 36 mg of mercury/kg of heart weight. Rapid cardiac asystole resulted from doses above 75 mg of mercury/kg of heart weight. Preliminary BAL, ascorbic acid, and thiamin, in descending order of efficacy, raised the blocking and cardiolethal dosages of mercuhydrin, and the same drugs administered following the mercurial compound partially reversed the cardiac effects measured. Theophylline ethylene diamine and epinephrine exhibited lesser cardio-protective activity. Magnesium sulphate and niacin had none.

The authors are indebted to Mrs. J. E. Johnson of the Department of Biological Chemistry for pH determinations of the perfusion fluids and to Dr. W. J. Wingo, formerly of the Department of Biological Chemistry, for checking the degree of solubility of BAL in aqueous solutions.

Received July 20, 1949. P.S.E.B.M., 1949, v72.

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Inhibition of the Urinary Excretion of 17-Ketosteroids by Caronamide. (17505)

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(Introduced by David K. Miller)

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The renal excretion of penicillin has been effectively inhibited by the concomitant administration of the drug, Caronamide (4′-Carboxyphenylmethanesulphonanilide)(1). Caronamide does so by inhibiting the transport mechanism responsible for the tubular excretion of penicillin.(2) It has been demonstrated that caronamide inhibits also the excretion of paraaminohippurate and phenolsulphonphthalein but has no effect on the glomerular filtration rate, the Tm of glucose or arginine, or the clearance of urea or sulfonamides.(3) It was thought to be of interest to study the effect of this compound on the urinary excretion of 17-ketosteroids in man.

Material and methods. The subjects studied were hospitalized ambulatory patients receiving the routine hospital diet. At no time during the study was any patient under any unusual "stress." Twenty-four-hour urine collections were checked for accuracy by creatinine determinations. Determination of creatinine was by the method of Folin(4) and of 17-ketosteroids by the method of Fraser *et al.*(5)

Exp. 1. After a control period of 2-3 days, during which 17-ketosteroid levels were determined, 1 female and 3 male patients received 24 g of caronamide* in divided doses per 24 hours. The female received the drug only 2 days; the males for longer periods. Each male had some degree of gastric distress toward the end of the drug period. Each subject had a sharp drop in the 17-ketosteroid level in the urine during the administration of drug, followed by a return to normal after a slight lag period (Fig. 1). This "lag" is probably due to continued elevated levels of caronamide due to accumulation of the drug, but since no blood caronamide levels were performed it is impossible to prove this point.

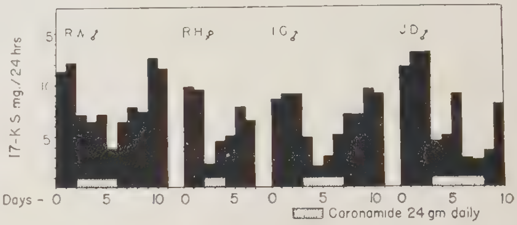

FIG. 1.

The inhibition of 17-ketosteroid excretion by the administration of caronamide. The sudden rise in steroid excretion in the case of Patient J.D. on day 6 of the study was apparently due to the patient's forgetting to take all his drug on this day, which he admitted on questioning.

Exp. 2. The injection of testosterone into the human is accompanied by an immediate increased excretion of 17-ketosteroids.(6) An experiment was devised to attempt to determine whether this increased excretion could be modified by caronamide administration.

Patient I.G., a eunuchoid male who had been studied in Exp. 1, was given a single injection of 80 mg of crystalline testosterone in aqueous suspension† after a 48 hour control period (Fig. 2). This was followed by a sharp increase in 17-ketosteroid output. Five days later, after the patient's steroid excretion

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* Caronamide (Statacin) was kindly supplied by Dr. W. P. Boger, Sharpe & Dohme Co., Glenolden, N.J.

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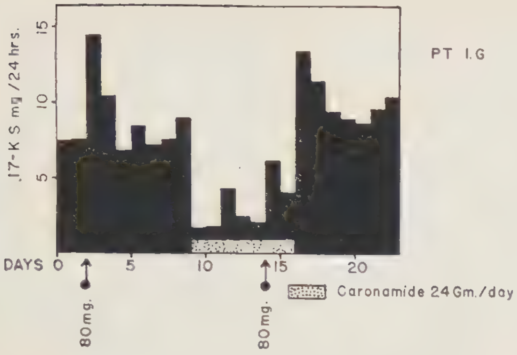

FIG. 2.

The modification of the expected sharp rise in 17-ketosteroid excretion following the intramuscular injection of testosterone by concomitant administration of caronamide.

became stabilized again, he was given 24 g of caronamide in divided dosage per 24 hours for 7 days. On the fifth day of this period he was given another injection of 80 mg of testosterone. The excretion of 17-ketosteroids following the injection of testosterone while the patient was receiving caronamide was distinctly less than that following the previous injection when the subject was not receiving the drug. However, when caronamide was discontinued 48 hours following the second injection, there was an immediate increase in steroid excretion.

Discussion. The data in Exp. 1 would seem to indicate that in sufficient dosage, caronamide will inhibit the excretion of endogenously manufactured 17-ketosteroids. The apparent absence of any "rebound" after the discontinuance and elimination of the drug might suggest increased degradation of these substances to non-17-ketosteroid derivatives when their natural avenue of excretion is blocked, or their alternate elimination by some other excretory route.

In reference to Exp. 2, it should be stated that in data not herein presented, we have evidence that caronamide must be given in sufficient dosage to effectively block renal tubular function if the excretion of administered testosterone is to be modified effectively. This has been shown true for penicillin also.(7) It is of some interest that the immediate, expected sharp rise in 17-ketosteroids following the injection of testosterone during the drug period was modified considerably, but that such an increase did occur following the cessation of the drug 48 hours later. This might suggest that the degradation products of administered testosterone are not further degraded rapidly when their natural pathway of excretion is blocked, and that renal tubular excretion may possibly represent their selective excretory route. It would also seem logical to assume from Exp. 1 and 2 that 17-ketosteroid excretion involves a renal tubular mechanism very similar to that for para-aminohippurate, phenolsulphonphthalein and penicillin. Further data must be obtained to substantiate these theories.

Conclusions. Caronamide (4′-carboxyphenylmethanesulphonanilide) has been found to inhibit the excretion of 17-ketosteroids in man. In effective dosage it will likewise apparently modify the expected rise in 17-ketosteroids following the intramuscular injection of testosterone.

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Received October 24, 1949. P.S.E.B.M., 1949, v72.

Antimalarial Activity of Aureomycin Against *Plasmodium gallinaceum* in the Chick. (17506)

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From the Laboratory of Tropical Diseases, National Institutes of Health, Bethesda, Md.

During the routine testing of several antibiotics for antimalarial activity, aureomycin was found active against *Plasmodium gallinaceum* in the chick. This is the first demonstration that aureomycin has activity against any malarial parasite.

Action against erythrocytic parasites (A-1 test).(1) This series of experiments was designed to test for action against erythrocytic parasites. Week-old New Hampshire Red chicks were inoculated intravenously with 16×10^6 parasitized erythrocytes. Treatment was begun 4 to 5 hours before inoculation and continued twice daily for 4 days. On the morning of the 4th day (day of inoculation is day 0) blood smears were examined and estimations made of the number of parasitized erythrocytes per 10^4 erythrocytes. In the first test aureomycin,* dissolved in water, was administered by injection into the pectoral muscles at a dose of 80 mg/kg. This regimen resulted in a significant reduction in parasitemia (Experiment 1, Table I). The chicks gained weight normally and there was no evidence of tissue damage at the site of inoculation. In Experiments 2, 3, 4 and 5 the aureomycin was administered orally. As shown in Table I, 75 mg/kg in all instances resulted in a pronounced reduction in parasitemia. Per unit weight the drug is thus approximately one-fourth as effective as quinine.(1)

TABLE I.
Activity of Aureomycin Against Blood-Induced *Plasmodium gallinaceum* in the Chick.

Exp. No.	Dosage of aureomycin mg/kg b.i.d. for 4 days	Parasitized r.b.c. in treated birds as compared with controls (%)
1	80*	12†
2	80	39†
3	75	4†
	150	6†
4	37.5	80
	75	33†
5	18	69
	37.5	63
	75	44†
	150	16†

* Intramuscular; all other dosages oral.

† The probability of this reduction in parasitemia occurring by chance is less than one in one hundred.

Action against early exoerythrocytic parasites (A-2 test).(1) The first test for causal prophylaxis included 30 chicks, New Hampshire Reds, one week old, divided into 3 groups of 10 birds each. Each chick received one mosquito equivalent of sporozoites injected into the pectoral region.(2) Treatment was begun 4 to 5 hours before inoculation and continued twice daily for 4 days. Group A received aureomycin orally at a dosage of 75 mg/kg twice daily, group B received 150 mg/kg twice daily, and group C served as the control. Blood smears were examined daily from the 7th through the 20th day, then every other day through the 35th day after inoculation. All control birds had detectable erythrocytic parasites between the 7th and 9th day after inoculation. Seven of the 10 birds in group A developed infections, the first on the 9th and the last on the 15th day after inoculation; in 50% of the group patency was delayed for 6 days beyond that of the controls. In group B, only 5 of the 10 birds developed patent infections, the first on the 11th day and the last on the 22nd day after inoculation. Whereas 9 of the 10 control birds died of an overwhelming exoerythrocytic infection (days 9 to 15), only 5 of the 20 treated chicks died as a result of either

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1. Coatney, G. R., and Sebrell, W. H., in Wiselogle, F. Y., *A Survey of Antimalarial Drugs, 1941-1945,* J. W. Edwards, Ann Arbor, Mich., 1946.

* Aureomycin was kindly supplied through the courtesy of Doctor J. M. Ruegsegger of the Lederle Laboratories Division, American Cyanamid Company.

2. Coatney, G. R., Cooper, W. C., and Trembley, H. L., *Am. J. Hyg.,* 1945, v42, 323.

exo- or endoerythrocytic infection during the 35-day observation period.

The second experiment included 45 week-old New Hampshire Red chicks, divided into 3 groups of 15 birds each. Each chick received 0.01 mosquito equivalent of sporozoites injected into the pectoral region. The treatment schedule, dosage of aureomycin for each of the 3 groups (A, B and C) and blood smear examination was exactly the same as described for the preceding experiment. In the control series, all of the chicks had detectable parasitemia 8 to 10 days after inoculation. Only 4 of the 15 chicks in group A and 2 of the 15 chicks in group B developed malaria. Whereas 11 of the 15 control chicks died of an overwhelming tissue infection, only 1 of the 30 treated chicks died during the observation period of 35 days.

Action against late exoerythrocytic forms. This experiment included 15 New Hampshire Red chicks one week old at the time of inoculation with 0.01 mosquito equivalent of sporozoites of *P. gallinaceum.* Five chicks were given aureomycin orally at 100 mg/kg twice daily for 7 days beginning at the time parasites were first detected in the blood, which in this test was 9 or 10 days after inoculation. The other 10 birds were kept as controls. On the 13th day after infection 9 of the 10 control chicks were dead and autopsies of their brains showed an overwhelming exoerythrocytic infection. (The remaining control chick survived until sacrificed 31 days after infection; no exoerythrocytic parasites were demonstrable). One of the 5 aureomycin-treated chicks died of an exoerythrocytic infection after 2 doses of the drug. The other 4 treated chicks survived until sacrificed 31 days after inoculation and no exoerythrocytic parasites were demonstrable at autopsy.

Although radical cures were not obtained, since erythrocytic parasites were demonstrable beginning 4 to 8 days after cessation of treatment, aureomycin was able to prevent death from exoerythrocytic forms, a property previously exhibited only by certain 8-aminoquinolines (pamaquine and pentaquine), chlorguanide (paludrine) and sulfadiazine.(3)

Summary. Aureomycin acted as a causal prophylactic and was therapeutically active against erythrocytic parasites and late exoerythrocytic forms of *P. gallinaceum* in the chick.

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3. Greenberg, J., Trembley, H. L., and Coatney, G. R., *Am. J. Hyg.*, in press.

Received October 25, 1949. P.S.E.B.M., 1949, v72.

Aureomycin in Experimental Chesson Strain Vivax Malaria. (17507)

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Aureomycin has been given a limited trial against sporozoite-induced Chesson strain vivax malaria in white male volunteers, and the results, although still incomplete, warrant a brief progress report. The procedures used were those employed throughout our program of screening speculative antimalarial drugs.(1) Very high dosages of aureomycin were given, approaching the range found by Coatney *et al.*(2) to be effective against *Plasmodium gallinaceum* in the chick.

Protective action. Two volunteers were each given 8 g of aureomycin* per day (1 g every 3 hours) on the day of bites by 10 heavily infected *Anopheles quadrimaculatus* mosquitoes, and for 6 days thereafter. They did not develop patent malaria until 30 and 31 days after exposure, whereas 4 controls

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1. Coatney, G. R., Cooper, W. C., and Ruhe, D. S., *Am. J. Hyg.*, 1948, v47, 113.

2. Coatney, G. R., Greenberg, J., Cooper, W. C., and Trembley, H. L., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 586.

* Aureomycin was furnished by courtesy of Doctor Stanton M. Hardy, Lederle Laboratories Division, American Cyanamid Company.

TABLE I.

Effect of Aureomycin on 4 Primary Attacks of Chesson Strain Vivax Malaria in Volunteers Bitten by 10 Infected Mosquitoes on 30 August. 1949.

	Volunteer No. 248			252			253			254		
Date, Sept.	Parasites, 5000 WBC	Peak fever, °F	Aureomycin (g)	Parasites, 5000 WBC	Peak fever, °F	Aureomycin (g)	Parasites. 5000 WBC	Peak fever, °F	Aureomycin (g)	Parasites, 5000 WBC	Peak fever, °F	Aureomycin (g)
11	<10	—	—	0	—	—	<10	—	—	0	100.2	—
12	<10	102.4	—	<10	—	—	0	104.0	—	0	105.2	—
13	200	99.4	—	100	98.4	—	230	105.0	—	190	100.6	—
14	140	104.2	8.0	130	103.6	—	190	105.4	4	140	105.2	4
15	3010	99.2	8.0	2310	99.2	8.0	4420	105.0	4	940	102.8	4
16	1890	104.0	7.3	780	104.4	8.0	2350	105.6	4	810	106.2	4
17	5430	99.6	6.6	6520	99.6	6.6	10,120	103.0	4	2380	103.2	4
18	4480	103.2	0.3	3350	104.6	2.3	4100	105.4	4	700	106.4	4
19	400	99.2	1.5*	890	98.8	1.5*	50	99.2	1.5*	40	99.6	1.5*

* Aureomycin was discontinued and quinine therapy instituted after the blood smears of September 19th had been made but before their examination.

bitten by the same mosquitoes had patent infections beginning 12 to 14 days after exposure. In 92 similarly infected controls of previous tests positive blood smears appeared 10 to 16 days after exposure (mean 12.0 ± 0.15). The delay in the aureomycin-treated subjects is interpreted as indicative of antiplasmodial action although complete prophylaxis was not attained.

Therapeutic action. The 4 controls from the foregoing experiment were treated with aureomycin. The original plan was to give 2 patients 8 g per day for 7 days and 2 patients 4 g per day for 14 days. The higher dosage caused nausea and vomiting in patients with active malaria, so that at times administration by vein was necessary. As shown in Table I, parasite densities in the blood increased and fever continued unabated until the 5th to 6th day of treatment, when the parasite counts in all subjects dropped precipitously. Unfortunately, because of the apparent lack of response, quinine sulfate therapy was begun on September 19, *after* the drop in parasite counts had occurred, but before the slides had been examined. This prevents knowledge of how soon aureomycin alone might have resulted in negative blood smears. The point is academic, however, because the action against erythrocytic parasites, although significant in primary attacks, is far too slow for practical usefulness. Relapses occurred in all of the 4 subjects within 10 to 14 days after the completion of quinine therapy (2 g per day for 7 days). Aureomycin, therefore, does not eradicate the fixed-tissue stage of *P. vivax*.

Summary. Aureomycin, although not a causative prophylactic against the Chesson strain of *P. vivax*, delayed the appearance of malaria in volunteers for at least 2 weeks as compared with controls. When given therapeutically in high dosage, the antibiotic had a delayed action against erythrocytic parasites, too slow to be of practical value. Infections were not cured.

Received October 25, 1949. P.S.E.B.M., 1949, v72.

Influence of Time of Ingestion of Essential Amino Acids upon Maintenance of Nitrogen Balance.* (17508)

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Accumulating evidence supports the assumption that all of the essential amino acids must be available in the diet simultaneously for growth or weight gain.(1-4) Previous experiments from this laboratory(3) demonstrated that protein-depleted rats did not gain weight when 2 incomplete essential amino acid mixtures (which when mixed constituted a complete mixture) were offered in rotation even an hour apart. However, because of the abrupt loss of appetite resulting from feeding rations deficient in one or more essential amino acids,(5) it was difficult to ascertain how much of the recorded loss of body weight was due to inanition and how much to failure of supplementation of one deficient mixture by the other. Similarly poor food consumption may have influenced the results of others who have reported similar experiments in the weanling rat. (1,2) In view of the theoretical and practical importance of this problem to man, whose appetite apparently is not reduced so greatly by the absence of a complete mixture of the essential amino acids, the present experiment was performed. It was planned so that the alternate ingestion of 2 incomplete essential amino acid mixtures, which together contained all 9 amino acids essential for growth and maintenance of the rat,(5-8) could be accomplished while all food constituents were being supplied at an adequate level.

Plan. Two groups of 6 adult male Sprague-Dawley rats of comparable weight and age were force fed all of their food at 12 hour intervals for 14 days. For 7 days the first group received alternately ration 5A containing 5 of the 9 essential amino acids and ration 4A containing the other 4 essential amino acids. During this period the second group received at each feeding a complete ration

TABLE I.
Amino Acid Composition of Mixtures Force Fed Normal Rats.

	Rations		
Amino acids	MAA	4A	5A
	g per 100 g of dry ration		
DL Isoleucine	.66	1.32	—
L (+) Lysine HCl	.16	.32	—
DL Phenylalanine	.15	.31	—
DL Tryptophane	.06	.11	—
L (+) Histidine HCl	.08	—	.15
L (+) Leucine	.21	—	.41
DL Methionine	.19	—	.37
DL Threonine	.27	—	.55
DL Valine	.51	—	1.03
Total essential	2.29	2.06	2.51
DL Alanine	.58	.58	.58
L (+) Arginine HCl	.51	.51	.51
DL Aspartic acid	.65	.65	.65
L (—) Cystine	.04	.04	.04
L (+) Glutamic acid	2.44	2.44	2.44
Glycine	.05	.05	.05
L (—) Tyrosine	.66	.66	.66
Total non-essential	4.93	4.93	4.93

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* The research which this paper reports was undertaken in cooperation with the Navy Department Office of Naval Research. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department. The work has been aided, also, by the National Livestock and Meat Board, and by the Douglas Smith Foundation for Medical Research of the University of Chicago.

1. Berg, C. P., and Rose, W. C., *J. Biol. Chem.*, 1929, v82, 479.

2. Geiger, E., *J. Nutrition*, 1947, v34, 97.

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5. Frazier, L. E., Wissler, R. W., Steffee, C. H., Woolridge, R. L., and Cannon, P. R., *J. Nutrition*, 1947, v33, 65.

6. Rose, W. C., *Phys. Rev.*, 1938, v18, 109.

7. Benditt, E. P., Humphreys, E. M., Straube, R. L., Wissler, R. W., and Steffee, C. H., *J. Nutrition*, 1947, v33, 85.

8. Wissler, R. W., Steffee, C. H., Frazier, L. E., Woolridge, R. L., and Benditt, E. P., *J. Nutrition*, 1948, v36, 245.

TABLE II.
Nitrogen Balance and Weight Changes in Rats Force Fed Incomplete Amino Acid Rations Alternately.

		1st 7 days			2nd 7 days		
Animal No.	Wt, g	Diet	Wt gain or loss, g	N bal., mg	Diet	Wt gain or loss, g	N bal., mg
1	325	4A, 5A alternated every 12 hr	—13	—303	MAA every 12 hr	— 1	+ 37
2	328		—13	—214		— 2	+ 83
3	306		— 7	—328		— 1	+ 15
4	313		—15	—226		— 2	+ 85
5	305		— 9	—264		+10	+ 65
6	322		—15	—276		+ 5	+ 91
Avg	317		—12	—269		+ 2	+ 63
7	332	MAA every 12 hr	— 8	+ 33	4A, 5A alternated every 12 hr	—10	—285
8	319		— 2	+ 50		—12	—225
9	322		— 2	+ 7		—13	—316
10	333		± 0	+ 41		—17	—241
11	317		— 2	+ 9		—12	—201
12	308		— 1	+ 71		—13	—301
Avg	322		— 3	+ 35		—13	—262

(MAA) containing the 9 essential amino acids. The rations were prepared so that all of the rats would receive equal quantities of all amino acids as well as the other food constituents every 24 hours, the only difference being the time of ingestion of the essential amino acids. During the second 7 day period the rations were interchanged so that the first group received the complete mixture and the second group received the alternating incomplete rations. Nitrogen balances and weight changes were measured for each 7 day period.

Rations, method of feeding and nitrogen balances. The quantities of amino acids in the 3 rations are shown in Table I. The quantities in the complete ration (MAA) were derived from data recently obtained in this laboratory.(9) They represent the minimum quantities of each essential amino acid necessary just to maintain weight equilibrium when fed at the daily level of approximately 3.6 g of amino acids (1.15 g essential and 2.45 nonessential) per kg of body weight and about 1200 calories per M^2 of surface area. The concentrations of essential amino acids in each of the other rations (5A and 4A) were double those in MAA in order to make the total daily intakes equal. The remainder of each of the rations in the "dry" form was made up of dextrin 64%, corn oil 4%, ruffex 5%, salt mixture 4%, water 16% and vitamins in quantities previously reported.(5) Each of the "dry" rations was diluted with additional water to make a thin soup which was thoroughly mixed in a Waring Blendor and fed in 13 ml quantities each feeding. This quantity supplied about 3.84 g of amino acids per kg and 1280 calories per M^2 of surface area per 24 hours. The rations were force fed by the method of Shay and Gruenstein.(10) The rats were adapted to this method of alimentation during a preliminary 6 day period by force feeding gradually increasing quantities of the MAA ration to each rat. Nitrogen balances were determined by methods previously described.(8) Fecal and urinary nitrogens were determined separately. Nitrogen intake was estimated by means of Kjeldahl determinations on multiple aliquots of the diluted rations.

Results. A brief summary of the experimental results is presented in Table II. The ingestion of the two incomplete amino acid mixtures at 12 hour intervals led to a weight loss of about 2 g per day per rat and an average negative nitrogen balance of approxi-

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9. Benditt, E. P., Woolridge, R. L., Steffee, C. H., and Frazier, L. E., unpublished data.

10. Shay, H., and Gruenstein, M., *J. Lab. and Clin. Med.*, 1946, v166, 429.

mately 260 mg for the 7 day period. On the other hand the rats receiving the complete ration each feeding almost maintained their weights and showed slightly positive nitrogen balances. This latter finding confirms the conclusion that the MAA ration is barely adequate to maintain nitrogen balance and weight when fed at the calorie level of $1200/M^2$. The results were essentially similar in each period. Thus the two sets of data gained by interchanging the diets after 7 days act as mutual checks and minimize the influence of the factors of time and animal variation. The fecal nitrogen values were not appreciably altered by the divided feeding of the essential amino acids. Furthermore, the total nitrogen ingested proved to be quite similar in the 2 groups during the 2 periods varying from 944 to 998 mg per rat for each 7 days. Therefore, the increased urinary nitrogen excreted by the rats receiving the essential amino acids in two feedings of incomplete mixtures at 12 hour intervals accounted almost entirely for the negative nitrogen balance which these animals exhibited. It is interesting to note that the difference between the average 7 day urinary nitrogens of the rats receiving the incomplete rations in rotation and those receiving the complete mixture each feeding was 359 mg. This is slightly greater than the total essential amino nitrogen of 316 mg/week which each rat received.

Discussion. These results suggest that the normal adult rat is unable to store even temporarily an incomplete mixture of amino acids for later use. Nor does there appear to be a means of drawing upon the abundant labile protein reserves(11) for a certain specific amino acid or certain amino acids which are absent from the diet. These data, therefore, confirm those reported for the protein-depleted rat(3) and suggest the rat's loss of appetite was probably a good indication of a lack of supplementation of the one deficient ration by the other. The only other nitrogen balance determinations done under conditions of delayed supplementation of which we are aware are those of Elman(12) who gave dogs tryptophane-deficient hydrolysates intravenously followed 6 hr later by tryptophane intravenously. Under these circumstances he noted a negative nitrogen balance. His experiments were not influenced by the speed of intestinal absorption or any problem of differential absorption of amino acids. Therefore it is of interest that the same type of result is obtained by oral force feeding in the rat if the stomach is allowed to empty before the supplementary ration is given. This inability of the normal adult animal to make up for an essential amino acid deficient even for a short time makes it even more important to avoid oral or parenteral feedings made up principally of incomplete proteins or amino acids especially during disease or convalescence. Further studies are needed to determine the source of the excess urinary nitrogen which is excreted under these circumstances.

Summary. The normal adult rat is unable to derive adequate nutritional value from an incomplete essential amino acid mixture which is supplemented 12 hours later by the missing amino acids even when attention is paid to strict equalization of the intakes of all food constituents.

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11. Madden, S. C., and Whipple, J. H., *Physiol. Rev.*, 1940, v20, 194.

12. Elman, R., PROC. SOC. EXP. BIOL. AND MED., 1939, v40, 484.

Received October 25, 1949. P.S.E.B.M., 1949, v72.

Capillary Circulation with Changes in Sympathetic Activity.* I. Blood Sludge from Sympathetic Stimulation. (17509)

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A study of the effects of sympathetic stimulation on the blood vessels of the conjunctiva of cats was undertaken incidental to some work on the physiological response of the nasal mucous membrane, but the findings seem of such general interest that it has been decided to report them separately. Suffice it to say here, that changes similar to those found in the blood vessels of the conjunctiva have been observed in the frontal sinuses and in the bullae of the middle ear as well as in the blood vessels of the fascia of the scalp. These latter regions are much more difficult to observe than are conjunctivae and there is, of course, much more tissue trauma in the preparation of these areas. This trauma can in itself produce blood sludging.(1)

Methods. Normal adult cats were anesthetized with veterinary nembutal, 30 mg per kilo intraperitoneally. The cervical sympathetic trunks were isolated for stimulation with a 60 cycle transformer resistance stimulator using 110 v. A.C. After tracheotomy, the cat's head was held firmly with a Grünfest head holder. The conjunctivae were made more accessible by slitting the external canthi with scissors and retracting the upper lids with traction sutures. The vessels of the eye were observed through a dissecting microscope (Leitz ×60-×120 magnification) using direct illumination from a dissecting lamp with two heat absorbing glass filters. The result is a clear view of venule and capillary circulation (Fig. 1). The arterioles are deeper and harder to see and are therefore not included in the diagram. The intact cervical sympathetic trunk was lifted gently from its bed and was stimulated on the observed side with sufficient current (¼ to ½ volt) to produce widening of the pupil.

Observations. Within a few seconds after cervical sympathetic trunk stimulation the arterioles and many of the venules of the conjunctiva can be seen to decrease in caliber. The red cells in the finer capillaries disappear or become very sparse. Meanwhile the flow in the venules and in at least some of the arterioles is markedly retarded, but in addition there are changes in the blood itself (Fig. 2). The blood cells cluster into clumps which appear identical with those described by Knisely *et al.*(2) as "blood sludge," *i. e.*, adherent aggregates of cells rather than ordinary rouleaux. As mentioned above, in some vessels the blood flow stops, but in a few venules, precapillaries, and capillaries it may reverse. This must be due to the arterio-venous shunts as has been described by Zweifach(3) and others. In somewhat larger vessels the flow continues, as a rule, in the same direction, but at a reduced rate. During stimulation almost all the blood cells in these vessels appear "clumped." In a few fields the clumps of blood cells can be seen squeezing out of a small constricted vessel into a larger vessel like cylinders of tooth paste coming out of a tube. These cylinders of clumped cells do not, as a rule, disintegrate on entering the next larger vessel in which blood flow continues more rapidly. After 15 seconds of stimulation the "sludging" effect increases for several seconds and then slowly disappears after 2 to 3 minutes with a return to normal of the caliber of most of those vessels which had been constricted. Later there seems to be dilatation of some of the vessels. At first, return to normal flow is observed in the larger vessels, then extending to the smaller vessels until all elements of the "sludge" have disap-

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* This work was supported by a grant from the United States Public Health Service.

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2. Knisely, Melvin H., Eliot, Theodore S., Bloch, Edward, and Warner, Louise, *Science*, 1947, v106, No. 2758.

3. Zweifach, Benjamin W., *Am. J. Anat.*, 1937, v60, 473.

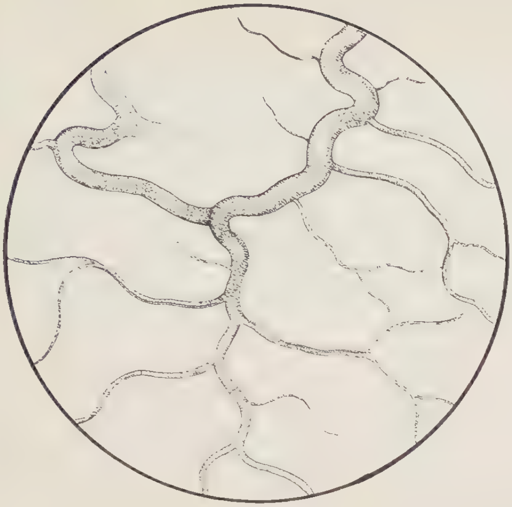

FIG. 1. (Left) Sketch of a normal conjunctival field × 100 approx.

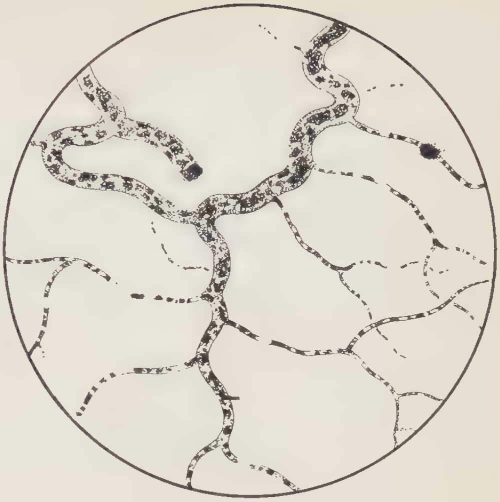

FIG. 2 (Right) Sketch of the same conjunctival field after cervical sympathetic trunk stimulation or injection of intravenous epinephrin. The smudge, upper right, is a small hemorrhage.

peared. The changes can be repeated several times if one is careful to avoid a hot illuminating light and the conjunctiva is prevented from drying with occasional drops of Ringer's solution. If the stimulation is continued for 60 seconds the sludging effect lasts five to six minutes and some clumping may be seen in the opposite eye.

Local application of 3 to 4 drops of epinephrine (1:10,000-1:50,000) produces a similar effect for a short time. A dilution of 1:1,000 usually produces prolonged stasis and a heavy sludge in most of the superficial vessels. One is immediately led to believe that the temporary hemostatic effect of epinephrin, so often used by surgeons, is not only due to contraction of the vessels, but also due to the creation of the multiple small "sludges" and only in some cases by thrombi in the vessels affected by the drug. Thus we have two mechanisms rather than one in the creation of hemostasis with local epinephrin when applied topically. The release of the corklike masses of "sludge" with dilatation of the vessels as the effect of the drug wears off may be an additional factor in the rather temporary character of the hemostasis produced in this way. A generalized "sludging" effect is produced by intravenous injection of epinephrin. Depending upon the speed of injection and the strength of the solution (1:1,000,000-1:10,000) almost any degree of blood "sludging" can be produced in a few seconds. Minute doses produce the faintest demonstrable clumping of the red blood cells, but increasing the dosage produces more and larger aggregates of cells until there is complete arrest of blood flow in the peripheral and central circulation with death of the animal. Very occasionally there are changes in capillary wall permeability which permits a few red cells to escape into the tissues. (Fig. 2, upper right). The effect of Nor-adrenalin is similar to epinephrin, but the response is much more transient.

Intravenous injection of novocaine in small doses will often reverse the sludging process, at least for a time, but this varies with the concentration of novocaine and the speed of injection. Further elaboration of the above experiment in various parts of the body and the various diseases now being studied for "sludge" should be interesting and illuminating.

Since fright or anxiety can produce stimulation of the sympathetic system(4) and blood sludge can be produced by sympathetic stimulation and since "blood sludging" has already been observed in psychotic states(2) one can not help calling attention to the possible occurrence of "blood sludge" and its

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4. Cannon, Walter B., *Bodily Changes in Fear, Hunger, and Rage*, D. Appleton, 1929.

consequences from emotional stimulation of the sympathetic pathways in the many diseases thought to be psychosomatic.

Summary. Stimulation of the cervical sympathetic trunk, application of local epinephrin, and injection of epinephrin intravenously has been found to produce, not only the classical picture of decreased circulation from narrowing of the vascular bed, but also what looks like blood "sludge" within the vessels. It is suggested that this may be of interest to those studying small vessel circulation and "sludge" and may have applications in general surgery, general medicine, and psychosomatics.

The author was assisted in these studies by Drs. Robert D. O'Malley, William A. Jarrett, and Octa C. Leigh.

Received October 25, 1949. P.S.E.B.M., 1949, v72.

Quantitative Measurement of Cutaneous Fluorescein Fluorescence as Indicator of the Capillary Circulation.* (17510)

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When sodium fluorescein is administered intravenously, it rapidly enters the interstitial fluid compartment. Due to its strong green fluorescence, its presence can be readily detected even in low concentrations when the skin, mucosa, or the surface of organs are viewed in suitably filtered long wave UV in a darkened room. Recently the passage of fluorescein into the interstitial fluid spaces was described as a function of capillary permeability.(1,2) However, no conclusive experiments designed to demonstrate this relationship have appeared in the literature. Although the specific factors regulating the passage of the dye through the capillary wall are not known with certainty, it can be clearly demonstrated that the patterns of cutaneous fluorescence, with respect to time and intensity, strongly mirror the capillary status of the region observed. Thus, intracutaneous epinephrine prevents fluorescein fluorescence at the site of injection, whereas histamine, acetylcholine, heat, etc. augment fluorescence.(1-4) Further, the intensity of fluorescence is quantitatively related to the concentration of the intracutaneously administered drugs as well as the amount of intravenously administered dye.(1,3) On the basis of this cursory relationship between fluorescein fluorescence and vascularity, it seemed worthwhile to refine methods of accurately measuring fluorescence *in vivo* with the intention of providing an objective tool for the quantitative study of the capillary circulation in physiologically intact preparations. Although Lange and Krewer(5) and Crismon and Fuhrman(6) have measured cutaneous fluorescein fluorescence by quantitative methods, experience with components of their apparatus have shown that they lack sufficient sensitivity and control to warrant usefulness as precision detectors of minute changes in fluorescence.

The instrumentation and methods reported herein are a modification of a procedure used for the determination of fluorescence in solids,(7) operating in principle similar to that of the dermofluorometer.(5)

Description of the Apparatus. The fluorescence measuring device designated as the

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* Supported by a grant in aid from the Graduate School Research Board.

1. Bukantz, S. C., and Dammin, G. J., *Science*, 1948, v107, 224.

2. Lange, K., and Boyd, L. J., *Arch. Int. Med.*, 1944, v74, 175.

3. Schiller, A. A., *Fed. Proc.*, 1949, v8, 139.

4. Schiller, A. A., to be published.

5. Lange, K., and Krewer, S. E., *J. Lab. and Clin. Med.*, 1943, v28, 1946.

6. Crismon, J. M., and Fuhrman, F. A., *J. Clin. Invest.*, 1947, v26, 259.

7. Schiller, A. A., and Cecchini, L. P., Project NM 001 008, Report No. 3, 21 July, 1948, Naval Medical Research Institute, Bethesda, Maryland.

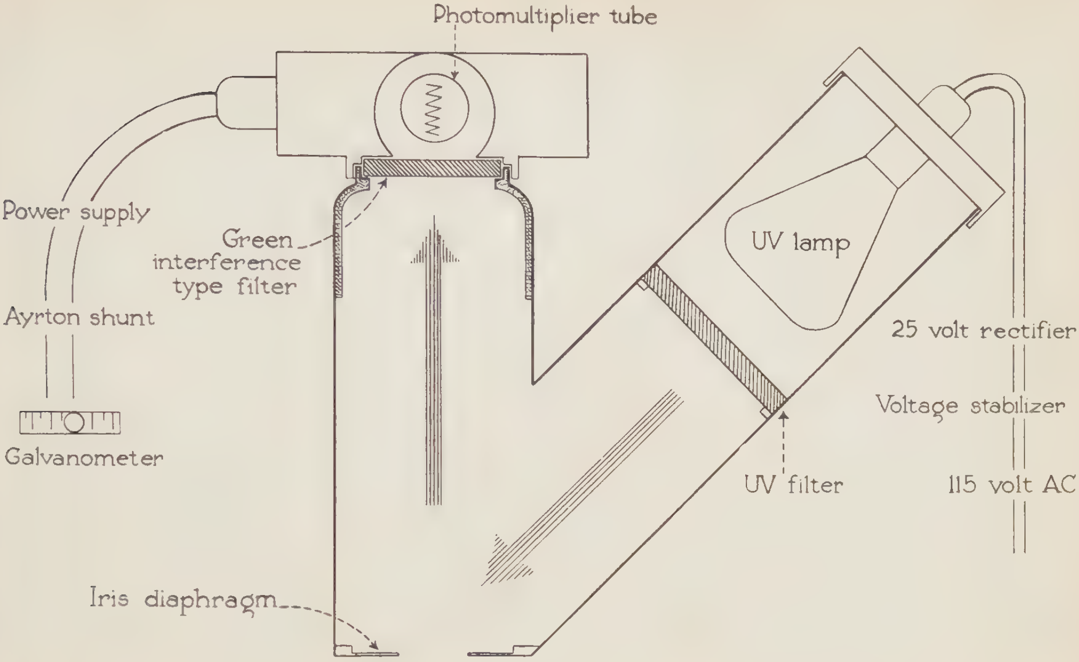

FIG. 1.
Fluorescence measuring device. (Not drawn to scale).

fluoro-illuminometer (F-I) consists essentially of a metal "Y" tube which serves as a rigid holder for a UV light source, photocell and filters (Fig. 1). The light source is a small 4 watt mercury vapor lamp[†] operating on stabilized rectified current of 20-24 volts. A Corning No. 5874 filter secured beneath the lamp absorbs the visible and transmits the UV radiation between λ 310-410 mμ, with a peak transmission at λ 365 mμ. The transmitted UV activates the specimen surface to fluorescence. The emitted fluorescence and an undetermined fraction of reflected UV passes normal to the specimen surface through an interference type filter[‡] which functions as a monochromator, transmitting a band of green light which peaks at λ 525 mμ. Thus, only the fraction of radiation from the specimen surface corresponding to the peak emission of fluorescein fluorescence reaches the multiplier phototube.[§] The photo current is read from a sensitive spotlight galvanometer.[‖] An Ayrton shunt[¶] included in the circuit permits the utilization of the full galvanometer scale over wide fluctuations of fluorescence intensity without altering phototube sensitivity. The high sensitivity of the photomultiplier tube more than compensates for the rather great transmission loss (60-70%) by the interference filter. Standardization of the apparatus is accomplished by the use of a stable reference material consisting of a 2″ x 2″ piece of green fluorescent glass[**] seated in a close fitting holder over a thin sheet of polished aluminum foil (Fig. 2). A primary

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[†] General Electric, 360 BL RP12.

[‡] Half width = 15±5 mμ; Farrand Optical Co., Inc., New York City.

[§] RCA 1P21.

[‖] Rubicon: Sensitivity = .002 μa/mm; Resistance = 4800 ohms; CDRX = 110,000 ohms. The phototube and housing, power supply, control unit and galvanometer were purchased as an integrated unit from Farrand Optical Co., Inc., New York City.

[¶] Leeds and Northrup No. 2164, 100,000 ohms.

[**] Canary glass No. 3750, Corning Glass Works, Corning, N.Y.

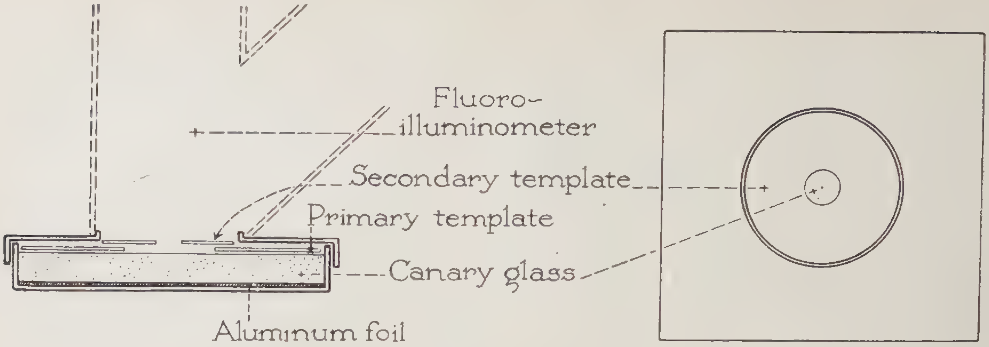

FIG. 2.
Reference standard. (Not drawn to scale).

template of black heavy paper with a central aperture 18 mm in diameter covers the entire upper surface of the canary glass. A metal cover with a 4.6 cm diameter lipped aperture fits snugly over the reference holder and primary template to accommodate the base of the F-I in a light-tight gravity seal. A number of circular secondary templates having central apertures from 1-10 mm in diameter are cut from thin copper sheeting and coated with dull black lacquer. These discs have a slightly smaller diameter than the aperture in the metal reference standard lid so that they may be easily removed by simple inversion of the reference holder.

Procedure. The UV lamp in the fluoroilluminometer is started approximately 10 minutes prior to the use of the instrument to ensure thermal stability and constancy of emission. The F-I is placed over the lipped aperture of the reference standard after insertion of a selected secondary template.†† The phototube is zeroed for dark current and the sensitivity is adjusted to read 100% on the galvanometer scale. Prior to the administration of the fluorescein, the F-I is placed over the skin sites the fluorescence of which is to be measured and pre-dye readings are made which are later subtracted from each post-dye determination, thus compensating for inherent skin fluorescence. After each measurement, or after a series of consecutive measurements, the F-I is again seated on the reference standard. A difference of more than 3% between the initial and final reference readings invalidates the measurement. If care is taken to ground and shield the equipment, and to select a UV lamp with stable characteristics, a series of 6 consecutive readings can be made in approximately one minute with reference variations of ± 1%. If the fluorescence from a small specific skin site, *e.g.* an intradermal wheal, is to be measured a blackened paper template about 2″ x 2″ with a central aperture corresponding to the diameter of the wheal is placed on the skin and the F-I centered on it. For more accurate centering on a discrete area, or when fluorescence is to be expressed as intensity per unit area, a prism finder head and iris diaphragm calibrated in terms of diameter can be fitted to the F-I. An accurate objective measurement of the circulation time of the dye is easily obtained by leaving the F-I on the skin while the dye is being injected. A sudden fluctuation of the galvanometer string indicates the exact moment of arrival of the dye at the skin site.

Results and discussion. The cutaneous vascular responses to a variety of chemical and physical agents employing the above procedure is under investigation. In most instances the vasoactive drug is prepared in serial dilutions and injected intradermally in the depilated abdomen of the rabbit, followed by the intravenous injection of sodium fluorescein (25 mg/kg in 5% aqueous solution). Fluorescent intensity over these sites

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†† The template selected is determined by trial and error. When the correct secondary template is chosen, it will usually be found that the entire range of fluorescence encountered can be read on the galvanometer scale without changing the shunt or phototube sensitivity.

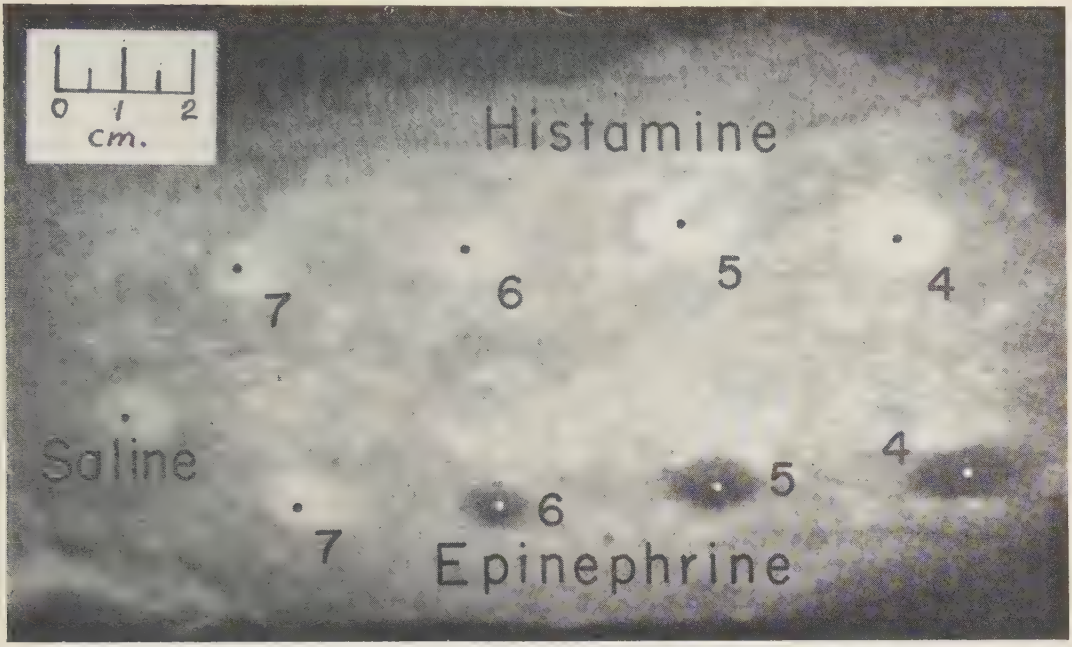

FIG. 3.
UV photograph of whealed rabbit's abdomen 9 min. after i.v. fluorescein (*cf.* Fig 4). The numbers refer to the concentrations of the drugs, *viz.*, 10^{-4}, 10^{-5}, etc. The black and white dots are artefacts to aid in identifying the center of each wheal.

is found to vary in a regular manner depending on the specific activity and the amount of the drug used as well as the anatomical location of the wheal. (Fig. 3). Time-intensity curves of fluorescein fluorescence are shown (Fig. 4) in which histamine and epinephrine were employed. It will be seen that generally the largest amount of epinephrine and histamine show the least and highest fluorescent intensities respectively, which is consistent with the known activities of these drugs. Also, the several dilutions of either drug produce a readily differentiable response at some point along the time axis which permits quantitative inter-comparison. The opaque colloidal dye, trypan blue, which is reputed to be an indicator of capillary permeability but which is poorly quantitated, has been compared with fluorescein in parallel experiments.(4) In every instance in which an agent has produced a positive trypan blue response, the intensity of cutaneous fluorescein fluorescence was well above the control level. The same agents in dilutions not capable of producing a positive trypan blue response did, however, show a relative increase in fluorescein intensity, implying that the fluorescein test is a sensitive indicator for intense as well as mild stimuli. It was noted frequently that agents which produced a positive trypan blue response, *viz*: concentrated histamine, heat, topical bromobenzene in ether, etc., were unphysiological stimuli and may have injured the capillary endothelium, suggesting that such a test may be invalid as an indicator of permeability changes in the physiological range.

Accepting the presently held hypothesis that trypan blue is an indicator of increased capillary permeability, and that fluorescein behaves similarly in that both dyes appear extravascularly in greater concentrations under identical experimental conditions, it may be deduced that the intensity of cutaneous fluorescein fluorescence may be dependent to some extent upon permeability changes. It is evident that a rigorous experimental demonstration must be provided before a permeability factor can be read into the cutaneous fluorescein intensity measurements in its pres-

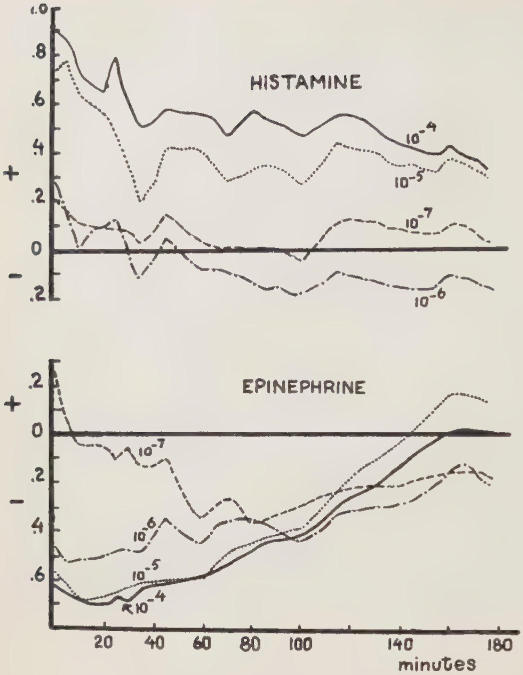

FIG. 4.

Fluorescein fluorescence time-intensity curves plotted as differences from the control saline wheal.

$$\text{Ordinate} = \frac{\text{fluorescence of drug wheal}}{\text{fluorescence of saline wheal}}. \quad + = $$

relative hyperfluorescence; — = relative hypofluorescence.

ent form. The rate of disappearance of fluorescein from the blood and the intact normal skin of the rabbit are closely parallel,(4,6) indicating that the altered time-intensity dye curves produced by the administration of vasoactive drugs must be a property of altered capillary hemodynamics and/or a change in the permeability of the capillary wall. The exact factors responsible for the rate of dye exchange through the capillary wall can only be determined by the simultaneous measurement of these independent variables. The estimation of cutaneous fluorescein fluorescence as described above quantitatively indicates the gross rate of dye exchange across the capillary wall (neglecting lymphatic participation), but does not offer insight regarding the responsible specific factor(s).

Summary. 1. A quantitative method for measuring cutaneous fluorescein fluorescence is described.

2. The intensity of cutaneous fluorescein fluorescence is shown to parallel the expected capillary responses to various concentrations of vasoactive drugs.

3. The procedure described can be used to obtain in physiologically intact, unanesthetized experimental animals and probably human beings, a quantitative, sensitive and objective estimate of the cutaneous capillary circulation under a variety of experimental conditions.

The author acknowledges his indebtedness to Mr. Carl Griesmeyer for the skillful and intelligent construction of the apparatus described in this paper.

Received October 25, 1949. P.S.E.B.M., 1949, v72.

Role of Vitamin B_{12} in Reproduction of Poultry.* (17511)

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Research of the past few years showed that dried cow manure and certain animal protein supplements contained an unknown factor (or factors) required by chickens for growth and reproduction.(1,2) When crystalline B_{12} became available, it was found to possess activity for chick growth equivalent to that of the unknown growth factor occurring in cow manure and liver extract.(3) The results

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* Presented as part of symposium on vitamin B_{12} and related factors, 116th meeting of American Chemical Society, September 22, 1949.

1. Rubin, M., and Bird, H. R., *J. Biol. Chem.*, 1946, v163, 387.

2. Whitson, D., Titus, H. W., and Bird, H. R., *Poultry Sci.*, 1946, v25, 143.

TABLE I.
Effect, on Hatchability, of Vitamin B_{12} Injections in Hatching Eggs.

				Type of injections			
Exp.	Class of embryo	Period of incub. at time of inj.	Level of B_{12} inj. per egg,* μg	None Hatchability of fertile eggs, %	H_2O %	B_{12} %	Increase or decrease over H_2O inj. eggs, %
215	—	10 days	1.25	70.0	47.8	71.4	+23.6
220	—	10 ”	1.25	75.0	82.8	80.0	— 2.8
228	—	Prior to incub.	0.50	—	74.4	83.3	+ 8.9
235	1	18 hr	—	83.5	—	—	
	2	”	0.50	—	70.0	88.9	+18.9
	3	”	0.50	—	0.0	80.0	+80.0
240	1	”	—	86.6	—	—	
	2	”	0.50	—	64.1	77.2	+13.1
	3	”	0.50	—	52.9	47.4	— 5.5
247	1	”	—	90.7	—	—	
	2	”	0.50	83.3	68.9	76.9	+ 8.0
	3	”	—	45.9	—	—	
252	1	”	—	85.5	—	—	
	2	”	1.0	71.4	40.5	43.5	+ 3.0
	3	11 days	1.0	—	43.8	71.4	+27.6

* Injected solution contained 25 μg of vitamin B_{12} per ml. An equivalent quantity of sterile distilled H_2O was injected per egg in the H_2O-treated groups.

of investigation of the role of vitamin B_{12} in reproduction are presented herewith.

Materials and Methods. Because of the limited quantities of crystalline vitamin B_{12} available, the effect of the vitamin was tested by injection into eggs rather than by administration to hens. All the hatching eggs used were laid by Rhode Island Red hens fed a diet deficient in vitamin B_{12}.(4) The treated eggs were wiped clean at the large end (position of air cell) with cotton saturated with alcohol. A hole was drilled through the cleaned shell. Injections were made by means of a hypodermic needle into the albumen. Then the treated eggs were sealed with scotch tape and set in incubation trays. In all experiments there were control eggs injected with distilled water and in some experiments there were additional untreated controls. The number of eggs was limited by the supply of crystalline vitamin, and although the hens were selected on the basis of a previous record of low hatchability, the sample of eggs used in the second experiment hatched too well to be suitable material.

Thereafter, eggs were selected for injection according to a method developed at this laboratory(5) for identifying eggs of low potential hatchability after 18 hours of incubation. By this method eggs are divided into 3 classes. Hatchability of fertile eggs in classes 2 and 3 is about 20% and 40% less, respectively, than in class 1. In most cases, eggs segregated in this manner were injected immediately after segregation, that is, after 18 hours incubation; but in one case the class 3 eggs were injected after 11 days incubation to permit removal of the considerable number of infertile eggs included in this class.

At hatching time all the chicks were wing-pedigreed and fed a 70% soybean meal basal diet(6) with and without a vitamin B_{12} concentrate. The chicks were reared in

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3. Lillie, R. J., Denton, C. A., and Bird, H. R., *J. Biol. Chem.*, 1948, v176, 1477.

4. Rubin, Max, and Bird, H. R., *Poultry Sci.*, 1947, v26, 309.

5. Olsen, M. W., *Poultry Sci.*, 1949, v28, 731.

6. Bird, H. R., Rubin, Max, and Groschke, A. C., *J. Biol. Chem.*, 1948, v174, 611.

electrically heated metal batteries with raised screen floors. Feed and water were supplied *ad libitum*. Body weights were recorded weekly, and feather observations at the termination of the experiments. All growth tests were of 6 to 12 weeks duration.

At the time of feathering observations, a feather scoring system was employed whereby those chicks with normal feathers were given the score "O." Those whose feathers were slightly frayed, thus having a rough appearance, were given a score of 1. Those whose feather tips were normal, but with an intermediate portion of the rachis free of barbs, were given a score of 2. Those with brittle, broken feathers were given a score of 3.

Results. Hatchability: The data on the effect of vitamin B_{12} injections on hatchability are summarized in Table I. Since hatching eggs of class 1, representing embryos of most advanced development, were characterized by high hatchability, none of these eggs was used for injection work. Due to the fact that a very small number of eggs of class 3 was available, none was used as a non-injected control. As indicated in Table I, in the last column, 7 out of a total of 10 lots of injected eggs responded favorably to B_{12}. Hence it is concluded that vitamin B_{12} is required for hatchability of eggs laid by hens fed a diet deficient in the vitamin. Of the 3 lots that did not respond, one, composed of unsegregated eggs (Expt. 220), showed too high a hatchability without vitamin B_{12} to permit any response, and another, the class 3 eggs in Experiment 240, constituted a small group in which the influence of unknown variables could easily have obscured the effect of the treatment.

Growth. The effects of B_{12} injections in hatching eggs upon growth of chickens are summarized in Fig. 1. When the chickens were fed a basal diet deficient in the vitamin, all those that hatched from B_{12}-injected eggs grew at a rate superior to that of the H_2O-injected and non-injected groups for a period of 12 weeks. Since no differences were found between the H_2O-injected and non-injected groups, the results from these two groups were combined. Fig. 2 shows the effect upon chick growth of different methods of administration of vitamin B_{12}. All the experiments in this case were of 6 weeks' duration. The first two curves are comparable to those shown in Fig. 1. The second two curves show that the growth response was greater for those chicks that received the supplemented diet than for those fed the basal diet alone when all chicks were hatched from eggs not injected with B_{12}. As is shown in the remaining curves, the combination of dietary supplement with egg injection of the vitamin produced the greatest response.

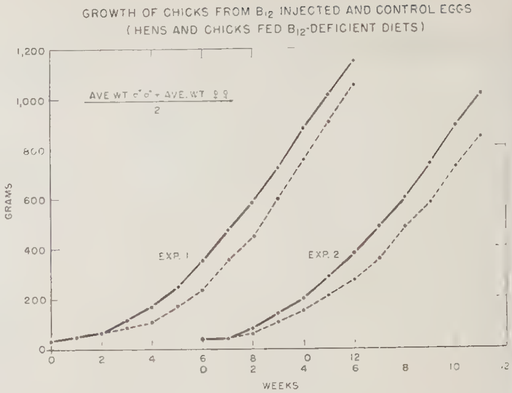

FIG. 1.

Growth of chicks from B_{12}-injected and control eggs. Hens and chicks were fed B_{12} deficient diets. Solid line, chicks from B_{12} injected eggs. Broken line, chicks from eggs not injected with B_{12}.

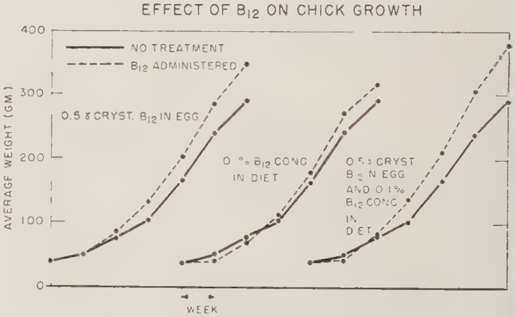

FIG. 2.

Effect on chick growth of vitamin B_{12} injected into egg, added to diet, or administered by both routes.

Viability and Feathering. It was reported previously(7) that chicks hatched from the eggs of hens fed a diet deficient in the then un-

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7. Bird, H. R., Rubin, Max, Whitson, Donald, and Haynes, S. K., *Poultry Sci.*, 1946, v25, 285.

TABLE II.
Effect of Vitamin B_{12} Injections in Hatching Eggs Upon Viability and Feathering of Chickens.

Exp.	Chick diet	Embryonic treatment	No. chicks		Mortality at 6 wk, %	Chicks with abnormal feathering, %	Feather score*
			At start	At end			
215	Basal	B_{12}	30	22	26.6	36.3	—
		No B_{12}†	34	13	61.7	84.6	—
220	Basal	B_{12}	20	17	15.0	23.5	0.35
		No B_{12}†	31	15	51.6	46.6	0.67
228	Basal	B_{12}	18	11	39.9	27.3	0.55
		H_2O	16	14	12.5	38.5	0.77
	+ B_{12}	B_{12}	17	11	35.3	9.1	0.09
		H_2O	16	9	43.8	44.4	0.55
247	Basal	B_{12}	10	7	30.0	0.0	0.00
		H_2O	24	15	37.5	53.3	0.60
	+ B_{12}	B_{12}	10	7	30.0	0.0	0.00
		H_2O	24	16	33.3	62.5	1.38
252	Basal	B_{12}	17	14	17.6	21.4	0.21
		H_2O	12	9	25.0	66.6	1.00
	+ B_{12}	B_{12}	15	11	26.6	27.2	0.45
		H_2O	12	10	16.6	30.0	0.60

* 0 = normal; 1 = slight abnormality; 2 = intermediate abnormality; 3 = extreme abnormality.

† Combined data from group receiving H_2O injection and group receiving no injection.

known factor suffered a high mortality during the first week of life. This mortality occurred even when the chicks were fed a supposedly complete diet but was corrected by correcting the deficiency in the hens' diet. It has also been observed that the progeny of hens fed such a deficient diet show abnormal feathering which is not corrected by adding supplements to the chicks' diet.

The effects of B_{12} injections in hatching eggs upon the viability and feathering of such chickens are summarized in Table II. With two exceptions, the mortality was greater for chicks hatched from H_2O-injected and non-injected eggs than for those hatched from B_{12} injected eggs, irrespective of chick diet. In experiments *215* and *220*, in which 1.25 micrograms were injected on the tenth day of incubation, the B_{12} injection reduced mortality very materially. Feathering of chicks hatched from B_{12}-injected eggs was superior to feathering of those from the H_2O-injected and non-injected eggs. As in earlier experiments addition of the vitamin to the diet of chicks hatched from deficient eggs was not effective in improving feathering.

Discussion. The data summarized here indicate the importance of vitamin B_{12} in reproduction of poultry, not only from the standpoint of hatchability but also from the standpoint of growth, viability and feathering of progeny. The effectiveness of a single dose of 0.5 to 1.25 μg of B_{12} injected into hatching eggs in enabling the progeny to maintain an advantage in growth over control chicks for a period of 12 weeks is remarkable and indicates the high potency of the vitamin per unit of weight. The fact that injection of vitamin B_{12} into deficient eggs reduced the mortality and improved the feathering of chicks and that these results were not achieved by supplementing the chicks' diet reemphasizes the importance of maternal diet and shows that deficient maternal diet may lead to irretrievable post-natal damage as well as pre-natal effects. Although vitamin B_{12} reduced mortality and improved feathering, it was not completely effective with respect to either. This might well have been due to the very limited dosage, but of course there exists the possibility that other dietary factors are involved.

Summary. When hatching eggs laid by Rhode Island Red hens fed a diet deficient in vitamin B_{12} were injected with crystalline vitamin B_{12}, the hatchability was improved. Among the chicks hatched from vitamin B_{12}-injected eggs, the growth rate was greater, the mortality lower and the feathering better than among chicks hatched from H_2O-injected and non-injected eggs.

Received October 26, 1949. P.S.E.B.M., 1949, v72.

Distribution of Chloramphenicol (Chloromycetin*) and its Metabolic Products Between Human Red Cells and Plasma. (17512)

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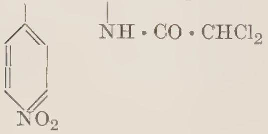
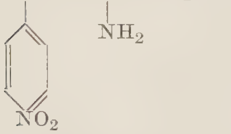
Following the administration of chloramphenicol to human subjects, 3 aromatic nitro compounds were identified as excretory products in the urine.(1) These were unchanged chloramphenicol (I), an amino diol produced from chloramphenicol by hydrolysis (II), and a conjugate of chloramphenicol with glucuronic acid (III):

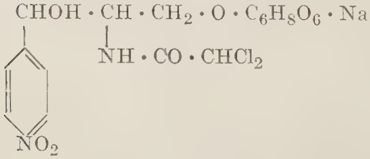
I. Chloramphenicol
D(—)*threo*-1-p-nitrophenyl-2-dichloracetamido-1,3-propanediol



II. Hydrolysis product
D(—)*threo*-1-p-nitrophenyl-2-amino-1,3-propanediol



III. Glucuronide derivative.



Of these compounds only I showed a significant degree of antibiotic activity. The glucuronide derivative III constitutes the major excretory product in human urine, although small amounts of I and II are also present.(1,2) The reduction of the nitro group is negligible in man, although considerable quantities of aryl amines may appear in the urine and feces of lower animals.(2) The data presented here summarize our observations on the distribution of I, II and III between the red cells and plasma of heparinized human blood.

Procedure. The chloramphenicol used in these experiments was a crystalline product isolated from fermentation sources.† The amino diol was prepared by hydrolysis of chloramphenicol,(3) and the glucuronide derivative was isolated from human urine in this laboratory.(1) Blood drawn from the antecubital veins of normal human subjects was treated with a few milligrams of powdered heparin (110 units per mg) to inhibit coagulation, and was used immediately. In a typi-

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* Parke, Davis and Company trademark for chloramphenicol.

1. Glazko, A. J., Dill, W. D., and Rebstock, M. C., *J. Biol. Chem.*, 1950, in press.

2. Glazko, A. J., Wolf, L. M., Dill, W. D., and Bratton, A. C., *J. Pharm. Exp. Therap.*, 1949, v96, 445.

† The chloramphenicol used in these experiments was kindly supplied to us by Dr. John Ehrlich and Dr. Fred Stimpert. The amino diol (II) was prepared from chloramphenicol by Dr. Mildred Rebstock and Dr. Harry M. Crooks.

3. Rebstock, M. C., Crooks, H. M., Controulis, J., and Bartz, Q. R., *J. Am. Chem. Soc.*, 1949, v71, 2458.

TABLE I.
Recovery Values for Known Amounts of Chloramphenicol, a Hydrolysis Product, and a Glucuronide-derivative of Chloramphenicol Added to Human Blood.

Compound	Conc. in blood (μg/ml)	Whole blood 1:20 (%)	Whole blood 1:40 (%)	Red cells 1:20 (%)	Red cells 1:40 (%)	Plasma 1:20 (%)	Plasma 1:40 (%)
I. Chloramphenicol	80	81	92	75	87	90	94
II. Hydrolysis product	53	90	96	94	96	94	94
III. Glucuronide derivative	125	89	90	89	95	92	99

Dilutions of 1:20 and 1:40 were made in 3% trichloracetic acid. The filtrates were analyzed for nitro compounds by the titanium reduction procedure. Values in the body of the table are given as per cent recovery, compared with those from aqueous solutions taken as 100%.

cal experiment a solution of purified I, II or III in physiological saline was added to heparinized blood and incubated at 38° with occasional shaking. Aliquots were withdrawn after 30 and 60 minutes, and a portion of each sample was centrifuged at 2100 r.p.m. for 15 minutes to separate the plasma and formed elements. The whole blood, plasma and red cells were then analyzed separately for aromatic nitro compounds, using the colorimetric procedure described elsewhere.(4) This method depends on reduction of the nitro group with titanous chloride, followed by diazotization and coupling with the Bratton-Marshall reagent.(5) The amount of color formed was found to be inversely proportional to the molecular weights of I, II and III. Samples were deproteinized with 3% trichloracetic acid using a sample dilution of 1:40. Colorimetric standards were set up with the same solutions used for the experiments, and corrections were made for recovery from whole blood, plasma and red cells as described in the next section. Packed cell volumes were determined on the original blood specimens and on diluted blood taken at each sampling period. This was done by centrifuging in Wintrobe tubes at 2500 r.p.m. for 15 minutes, reading cell volumes, and then recentrifuging to check the first reading. These figures were used for calculating the total content of nitro derivatives in the plasma and cells as a check on the analytical results.

Results and discussion. Data for the colorimetric recovery of known amounts of I, II and III added to whole blood, plasma and red cells are presented in Table I. The blood samples were diluted 1:20 and 1:40, using a final concentration of 3% trichloracetic acid, and filtrates were analyzed for nitro compounds by the titanous reduction procedure.(4) From these results it is evident that dilutions of at least 1:40 must be used for satisfactory recovery of nitro compounds in the presence of red cells. These values were used to correct the analytical data obtained in the distribution experiments. The distribution of I between red cells and plasma was studied by the addition of 1925 μg I in 6 ml of saline to 10 ml of heparinized blood. After standing at 38° with occasional shaking for 30 and 60 minutes, samples were removed for analysis as already described. In a similar manner, the amino diol compound (II) was studied by the addition of 640 μg II in 3 ml saline to 10 ml of blood from a different individual. The glucuronide derivative was also handled similarly, with the addition of 2325 μg III in 3 ml saline to 10 ml of heparinized blood. The results of these experiments are presented in Table II.

From these data it is evident that I and II are bound to a considerable extent by the formed elements of blood, while III is located almost entirely in the plasma with little or none present in the red cells. The binding is probably due to adsorption rather than slow diffusion through the cell membrane, since higher concentrations of nitro compounds are found in the red cells than would be expected on the basis of uniform distribution. This is also supported by evidence for the binding of

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4. Glazko, A. J., Wolf, L. M., and Dill, W. D., *Arch. Biochem.*, 1949, v23, 411.

5. Bratton, A. C., and Marshall, E. K., *J. Biol. Chem.*, 1939, v128, 537.

TABLE II.
Distribution of Nitro Compounds Between Plasma and Red Cells.

Compound	Theoretical conc. (µg/ml)	Incub. (min.)	Packed cell vol. (%)	Conc. of nitro compounds			Ratio: red cells/ plasma
				Plasma (µg/ml)	Red cells (µg/ml)	Whole blood (µg/ml)	
I. Chloramphenicol	120	30	32.6	100	153	123	1.53
		60	32.6	98	157	118	1.60
II. Amino diol hydrolysis product	49	30	31.1	39	61	47	1.56
		60	33.7	33	68	46	2.06
III. Glucuronide derivative	179	30	39.3	274	55	168	0.20
		60	40.6	290	38	172	0.13

Plasma, red cells and whole blood were analyzed for nitro compounds following equilibration at 38° for 30 and 60 minutes, as described in the text. Values were corrected for recovery from each fraction as indicated in Table I. The ratios of concentrations found in red cells and plasma are given in the last column.

I to serum albumin.(6) In most cases, the amount of drug accounted for by the separate analysis of plasma and cells fell within 5 per cent of the actual amount of drug added. The observations presented here may assist in the interpretation of published data on the renal excretion of chloramphenicol and its inactive metabolic products.(2) The excretion rate for the glucuronide derivative III is no doubt greater than if it were bound to the cellular elements of blood, while the excretion of unchanged chloramphenicol is probably retarded by adsorption onto the erythrocytes, which would lower the effective concentration of freely diffusible drug in the plasma. The binding of chloramphenicol on the red cells, and possibly on the cells of other tissues, may constitute a reservoir of antibiotic which is in equilibrium with the body fluids. The existence of a different mechanism for producing active chloramphenicol by enzymatic hydrolysis of the inactive glucuronide has been described elsewhere.(2) These two factors may well be responsible for the maintenance of therapeutic levels of chloramphenicol in the blood stream over long periods of time.

Summary. Chloramphenicol and one of its hydrolysis products appear to be partly bound to the cellular elements of blood, while the glucuronide derivative of chloramphenicol remains almost entirely in the plasma. Observations are also presented on the recovery of these aromatic nitro compounds from trichloracetic acid filtrates of plasma, cells and whole blood by colorimetric methods.

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6. Vandenbelt, J. M., cited by Smith, R. M., Joslyn, D. A., Gruhzit, O. M., McLean, I. W., Penner, M. A., and Ehrlich, J., *J. Bact.*, 1948, v55, 425.

Received October 27, 1949. P.S.E.B.M., 1949, v72.

A Simple Means of Producing Obesity in the Rat. (17513)

DWIGHT J. INGLE.

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The effects of overfeeding and of obesity have been little studied in the laboratory. Voluntary hyperphagia in rats has been stimulated by insulin(1) and by lesions in the hypothalamus.(2) In this laboratory, we have produced marked obesity in rats by

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1. MacKay, E. M., and Cullaway, J. W., PROC. SOC. EXP. BIOL. AND MED., 1937, v36, 406.

2. Hetherington, A. W., and Ranson, S. W., *Anat. Rec.*, 1940, v78, 149.

force-feeding.(3) There is a simple method for the production of obesity in the rat, new in the laboratory, but representing a principle known throughout the history of medicine and animal husbandry. The development of obesity is related to the level of energy output as well as to the level of food intake. Restriction of the activity of the rat combined with the *ad libitum* eating of a diet which is appetizing to the animal, is a reliable means of promoting gains in weight in excess of the values regarded as standard for albino rats.

Male rats of the Sprague-Dawley strain were maintained on a stock diet of Archer Dog Pellets until they reached a weight of 325 to 375 g. They were then transferred to cages of one-half inch mesh screen 9 inches long, 4½ inches wide and 4½ inches deep. They were given a fluid diet to eat *ad libitum* (Table I). The room temperature was 74° to 78°F. Six rats were studied under these conditions. All of them gained in weight more rapidly than active stock animals or active animals on the same diet. We have never recorded a weight greater than 550 g among active adult male rats on the stock diet nor have we recorded a weight greater than 600 g for similar active animals fed the fluid diet in the large series of animals which we have observed during the past 10 years. After all of the 6 inactive rats were in excess of 700 g weight, 4 were killed for tissue studies, one reached a weight of 946 g and died and the 6th rat attained a maximum weight of 1090 g at which time it began to lose weight slowly and was killed for tissue studies. The weight chart of this animal is compared with the record of a typical active rat on the stock diet in Fig. 1. Insofar as we are aware, this is the greatest weight that has been reported for an albino rat.

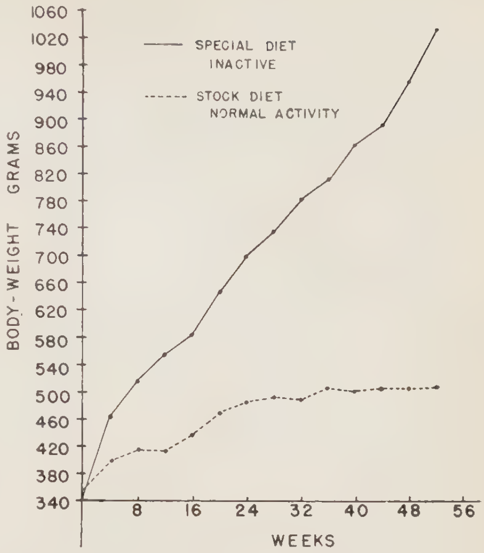

FIG. 1.
Changes in weight of two normal rats.

TABLE I.
Medium Carbohydrate Diet.

Constituent	g
Cellu flour (Chicago Dietetic Supply)	120
Osborne and Mendel salt mixture	40
Diet yeast (Pabst)	100
Wheat germ oil	10
Cod liver oil	10
	mg
Vit. K (2-methyl-1,4-naphthoquinone)	100
Mazola oil	200
Casein (Labco)	160
Starch	200
Dextrin	190
Sucrose	200
	cc
Water to make total of	2000

Summary. Obesity can be produced in the albino rat by restriction of activity and by the *ad libitum* eating of a diet which is appetizing to the animal.

Received October 27, 1949. P.S.E.B.M., 1949, v72.

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Action of Bacterial Toxins on Respiration of Chicken Erythrocytes.* (17514)

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In the first paper of this series (Hunter and Larsh)(1) a brief statement of the general problem which is being investigated in this laboratory was presented. The present experiments were designed to demonstrate what effects, if any, a number of bacterial exotoxins have on the functioning of cells as indicated by changes in respiration.

The toxins were obtained from the Lilly and Lederle Laboratories§ with the exception of *B. cereus*. This organism was grown in Bacto proteose peptone No. 3 broth and incubated at 37.5°C for 36-60 hours. The fluid suspensions were filtered through Seitz or Berkefeld filters and stored at 4°C with the relative humidity controlled at 95-100%.

A suspension of erythrocytes was obtained by centrifuging heparinized chicken blood. Equal volumes of cells and toxin, cells and heated toxin, cells and formalized toxin, and cells and preservatives (phenyl mercuric acetate or merthiolate) were mixed. Respiration measurements were made at 37°C, using a Warburg apparatus. One or 2 cc of the various cell mixtures were added to each Warburg vessel which contained 0.3 cc of 20% KOH in the center well. The time which elapsed from the mixing of the suspensions until the first reading was made varied from 15 minutes to an hour. Readings were started after a 10-minute period for temperature equilibration and were continued for varying periods of time up to 25½ hours. Sterile technics were used throughout and tests for contamination using agar slants and fluid thioglycollate were made at the end of each experiment.

The results are summarized in Table I, which also includes information concerning the relative strength of most of the toxins. It can readily be seen that formalized toxins are of no value as controls since formalin markedly inhibits the respiration of these cells. Except for the case of tetanal and streptococcal toxins, the heated toxins give essentially the same values as the Ringer Locke controls. Since the toxins were not highly purified, the conclusions which follow are tentative and may well have to be modified when experiments using purified toxins are available.

Summary. 1. Chicken erythrocytes were exposed to various bacterial toxins for several hours and the rate of oxygen consumption was measured at 37°C.

2. Toxins from *Clostridium perfringens* appeared to increase the rate of oxygen consumption initially even though they hemolyzed the cells. After several hours a decrease was observed.

3. Toxins from *Clostridium septicum* had little effect on the rate of oxygen consumption.

4. Toxins from *Clostridium tetani* apparently increased the rate of oxygen consumption for a period of more than 24 hours.

5. Toxins from *Bacillus cereus* had little effect, but slight acceleration of oxygen consumption was noted in most of the experiments.

6. Toxins from *Micrococcus pyogenes*, Var. *aureus* increased the rate of oxygen consumption during the first hour. This was followed by hemolysis of the cells and almost complete inhibition of respiration.

7. Toxins from *Corynebacterium diphtheriae*

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* This work was supported in part by grants from the Division of Grants and Research, U. S. Public Health Service, and the Faculty Research Fund of the University of Oklahoma.

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§ The authors are indebted to the Lilly Laboratories and to the Lederle Laboratories for supplying these toxins.

TABLE I.
Rate of Respiration of Chicken Erythrocytes in the Presence of Various Bacterial Exotoxins.

	μ L $O_2/10^9$ cells/hr												
	Ringer Locke			Heated toxin (75-82°C, 75 min.)			Formalized toxin (0.02 M formaldehyde 2 wk at 37°C + 2 mo. at 6°C)			Toxin or preservative			
Cells exposed to equal volumes of:	1st hr	2nd hr	3-5th hr	1st hr	2nd hr	3-5th hr	1st hr	2nd hr	3-5th hr	1st hr	2nd hr	3-5th hr	Remarks
1:20,000 phenyl mercuric acetate	9	10								9	11		No effect
1:10,000 merthiolate	9	10								9	10		,, ,,
Cl. perfringens—strength unknown	9	9	8*	11	11		3	3	2*	14	7	0*	Initial acceleration, partial inhibition; complete inhibition‡
Cl. septicum—160 MLD for mouse/cc	11	11		11	11		<1	<1		8	9		Little or no effect
Cl. tetani—300,000 MLD for guinea pig/cc	10	10	10†	13	14	13†	6	5		13	14	13†	Possibly slight acceleration
B. cereus—strength unknown	9	9		11	10		1			15	15		,, ,, ,,
M. aureus—15,000 dermal necrotic doses/cc	11	10	10	10	9	9	6		4	18	4	0	Initial acceleration, marked inhibition; complete inhibition‡
C. diphtheriae—800 MLD for guinea pig/cc	12	12	12	10	8		5		2–3	20	7	4–3	Initial acceleration, partial inhibition, marked inhibition
Str. pyogenes—strength unknown	11	11		3	3		<1	2		3	<1		Marked inhibition, almost complete inhibition

* 6–7 hr. † 3–4 hr. ‡ Possibly result of hemolysis.

increased the rate of oxygen consumption slightly during the first hour. This was followed by a gradual decrease in rate until after 11 hours, then there was complete inhibition.

8. Toxins from *Streptococcus pyogenes* markedly inhibited the rate of oxygen consumption although there was no hemolysis.

Received October 27, 1949. P.S.E.B.M., 1949, v72.

Studies on Proteinuria in the Rat. (17515)

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Most investigations of protein excretion in the urine have assumed that protein in quantity is an abnormal constituent of the urine. It has furthermore been commonly assumed that mammalian kidneys extract from plasma a protein-free fluid in each species. The existence of a normal proteinuria in the Slonaker strain of white rats has been described qualitatively by Addis.(1,2) Protein excretion has been studied by Smetana(3) by injecting dye-labelled foreign protein into the blood of several mammals and observed deposition of dyed particles within the lining cells of the proximal convoluted tubules. Oliver(4,5) used similar technics both *in vivo* and in perfused isolated kidneys, with similar results. Dock(6) injected Evans blue intraperitoneally in rats and subsequently observed dye deposition in the renal tubules. The present studies again demonstrate a normal proteinuria, both albuminuria and globulinuria, under a variety of environmental conditions, in the albino rat. The passage of plasma protein through the rat kidney is studied by labelling the native plasma protein with Evans blue, and examining, in fixed tissues, the disposition of the Evans blue-protein complex.

Procedures. A. *Protein determinations.* Urine was collected from rats, weighing from 180 to 250 g each, while in individual metal metabolism cages. During periods of urine collection, none of which was longer than 24 hours, no food was present in the cage. The animals studied included 14 male animals of the Wistar strain, on "Rockland Rat Diet", and located in a constant temperature room at 27° C; 12 animals, 8 males and 4 females, of the Sprague-Dawley-Holtzman strain, at ordinary room temperature, that had been on a diet of fortified powdered milk for a month; and 8 male rats of the Sprague-Dawley-Holtzman strain that had been subjected to a constant air temperature of 4° C for 3 months.

a) The urine was collected individually, centrifuged clear, and tested for protein content by heating and adding acetic acid. The precipitate so obtained was spun down washed and tested by the biuret test, xanthoproteic test, charring, and Millon test.

b) Qualitative protein fractionations of 72-hour urine specimens from 9 normal rats were performed. Urine was saturated with ammonium sulfate and a precipitate was interpreted as albumin. Globulin was precipitated by half saturation with ammonium sulfate. In both instances the precipitates were tested with protein reagents as above.

c) Quantitative protein measurements were made on 24-hour pooled urine from 5 normal male rats. Total protein was determined by precipitating with trichloracetic acid, centrifuging, dialyzing against distilled water at 5° C till chloride-free, and drying to con-

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stant weight at 95° C. Its globulin was determined by precipitating with equal parts of 4 M potassium acid phosphate solution (by the method of Butler),(7) centrifuging, and redissolving in physiological saline, then dialyzing against distilled water until chloride- and phosphate-free. At completion of the dialysis the globulin had reprecipitated. Albumin was determined by precipitating from the supernatant urine, after the globulin was precipitated, with an equal part of 20% trichloracetic acid, centrifuging, resuspending the precipitate in water, and dialyzing until chloride- and phosphate-free.

B. *Evans blue* (*T-1824*) *determinations.* Evans blue forms a complex with plasma proteins. This property is made use of in the present experiments to label plasma proteins in their passage through the kidneys. Rawson(8) ascertained that, at pH 7.4, in solutions of human or canine plasma albumin, up to 14 molecules of Evans blue are linked to one molecule of plasma albumin. The greatest stability of the dye-protein complex is attained up to ratios of 8 molecules of Evans blue per molecule of plasma albumin. At such concentrations, the dye is wholly and preferentially bound by the albumin fraction, and in increasing proportions as the concentration of the dye increases. If the dye concentration is increased sufficiently, the dye may also be bound by the globulin fraction, but preferentially by the alpha-globulin. That, in the equilibrium system between the protein-dye complex and the free dye and plasma protein, the concentration of free dye is infinitesimal, is indicated by the observation of Rawson(8) that ultracentrifugation of a solution of dye-protein complex centrifuges down a blue-stained layer and leaves an unstained supernatant. Trypan blue is also bound by plasma protein; this complex is more unstable than the Evans blue-protein complex. When the trypan blue-protein complex is dialyzed in a cellophane bag the trypan blue is separated from the protein and stains the cellophane. Evans blue, however, remains bound to protein under such circumstances, and leaves the dialyzing membrane colorless. The Evans blue molecule has 4 sulphonic-acid groups, and has a molecular weight of 960. The attachment of several such dye molecules to a molecule of plasma protein modifies the charge and the weight of the colloidal particle, and the addition of Evans blue to a solution of albumin has been found to lower appreciably the isoelectric point of the protein.(8)

A series of 8 male rats were given intracardiac injection of 0.5 ml of 5% Evans blue solution. The blood volume of the rat is reported as 4 ml per 100 g body weight,(9) of which approximately one half is plasma. Plasma protein concentrations are 5 to 7% (10) with about a 5 to 2 ratio of albumin to globulin.(10) It is readily calculated that, in the dose administered, all the dye combined with the plasma proteins. Starting 3 hours after administration of the Evans blue, urine was collected for 24 hours, observed qualitatively for color, and tested for protein with heat and acetic acid. The animals were then decapitated and autopsied. Kidneys were formalin-fixed in two series, unstained and stained with hematoxylin and eosin respectively, so as to facilitate study of both the course of the dye and of any possible renal damage that might have resulted from the injected Evans blue. In order to observe *in vitro* formation of the dye-protein complex in protein-containing urine, one drop of 5% Evans blue solution was added to the 24-hour urine sample of each of 4 untreated normal rats, and within two minutes this urine was tested for protein, as above.

Next, a second series of 13 male rats received intracardiac injections of 0.4 ml of 5% Evans blue. These rats were all decapitated, 7 rats at 10 minutes after injection of Evans blue; and 6 rats, 90 minutes after injection. Kidney slices from each of these rats were hardened in formalin solution for

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the preparation of frozen sections.

Results. A. *Urinary proteins.* Two male rats maintained in the constant temperature room at 27°C gave negative tests for urinary protein. The other 28 rats, including animals of the 2 strains studied, and animals maintained at the several above-mentioned temperatures and diets, showed proteinuria. The amounts of protein, as observed grossly by the relative degrees of turbidity or flocculation on treatment of the urine with heat and acetic acid, varied in all groups, except in the group acclimatized to cold, from a moderate turbidity to a heavy flocculent precipitate. Urine samples collected from the animals kept in the cold room all showed heavy flocculent precipitates. All urine specimens fractionated by ammonium-sulfate precipitation contained both albumin and globulin. The urinary proteins were determined as 6.6 mg of protein in an average urine quantity of 8 ml per 210 g rat per 24 hour period. As separately determined, 3.0 mg of globulin, and 3.3 mg of albumin were excreted per rat in this period. No attempt was made to estimate the variation of proteinuria in an individual, but there was an appreciable variation in the bulk precipitate in the same rat in several 24-hour qualitative protein determinations.

B. *Evans blue studies.* Several rats, not included in the numbers reported above, died within a few minutes of injection with Evans blue, presumably from cardiac or pulmonary injury. All the 24-hour urine samples of the first group of 8 rats injected with Evans blue were blue-stained. The urine of all these animals was positive for protein as it had been in each animal when tested before the dye injection. The protein precipitate in all instances showed blue, and, when allowed to settle, was found to have withdrawn the dye from solution, the supernatant retaining no visible tinge. Normal rat urines to which a drop of Evans blue was added *in vitro* had their proteins precipitated with heat and acetic acid; precipitates so obtained were blue, and the supernatant showed no blue stain. All injected animals were decapitated. On macroscopic examination, in 21 animals injected with Evans blue, the tissues were, to a greater or lesser degree, blue stained. The liver contained the most deeply dyed tissue. Kidney sections showed heavily stained cortical papillae which were sharply demarcated from the lightly tinted medullary tissue. Selective staining of the cortex was most intense in the animals killed after 24 hours, and least apparent in the specimens taken ten minutes after injection of the Evans blue. Microscopic studies of the kidney tissues of Evans blue-treated animals revealed several findings (see Table). All fixed hematoxylin and eosin stained sections taken from animals even 24 hours after Evans blue injection showed no morphological renal damage.

(a) In frozen kidney sections cut from animals killed 10 minutes after injection, blue-staining granules were observed, all superficially distributed in the cytoplasm (that is, toward the tubular lumen) of proximal tubular cells of many nephra. Several sections contained proximal tubular cells with a diffuse blue stain toward their luminal surface, as well as a similarly distributed, granularly disposed stain. The glomerular and capsular spaces were uniformly and distinctly clear of visible material, blue or otherwise. Blue-stained casts were found in the proximal tubules and in the collecting tubules of all specimens.

(b) In frozen sections of kidneys removed from animals killed 90 minutes after Evans blue injection, blue-staining granules were again observed in the cytoplasm of proximal tubular cells. Now, however, no diffuse distribution of the blue dye was apparent. Blue-staining granules in the cytoplasm were distributed superficially as well as more deeply throughout the cells, although the stained particles seldom approached the basement membrane, and were more concentrated toward the lumen. Cells in this group of sections contained appreciably more dye than in the ten-minute group. Also, the dyed particles were not merely in the shape of irregular granules of varying size as in the 10-minute sections, but frequently had a filamentous and often branching appearance, such as to suggest the possibility that the dye had become adherent to mitochondria.

(c) Kidney sections of animals killed 24 hours after injection received no post mortem

TABLE I.
Distribution of Evans Blue in Kidney Sections Taken 10 Min., 90 Min., and 24 Hr After Dye Injection.

Structures observed	10 min.	90 min.	24 hr
Blue-stained casts	+	+	+
Diffuse stain of superficial surface of tubular cells	+	—	—
Granular stain of tubular cells superficial	+	+	+
Luminal half of cells	—	+	+
Throughout cytoplasm	—	—	+
Stained granules in tubular cells showing various filamentous and rod-shaped forms	+	+	Indeterminate
Blue-stained arterial and arteriolar elastic membranes	+	+	+

stain, but were passed through paraffin to make permanent sections. These tissues showed the densest aggregations of intensely blue-stained particles, so numerous as to make any distinction between massed particles and single filaments very dubious. In these sections the dye was fairly evenly distributed throughout the cell, from luminal border to basement membrane (see Fig.). Again, in these sections, blue-stained casts were observed both in the proximal tubules and in the collecting tubules. Of much interest is the finding that all kidney sections contained areas of unstained proximal tubules which were estimated to be at a comparable level along the tubules as were intensely stained regions of other tubules in the same section. Hence it would seem probable that the dye-protein complex was filtered through the glomeruli in high dilution, and partly reabsorbed in the proximal tubules, possibly through the intermediation of mitochondria. Sections of the liver showed blue-dyed granules in the Kupfer cells and also showed blue-stained droplets of varying sizes in the cytoplasm of the liver parenchymal cells. Adrenal parenchymal cells and cardiac muscle fibers were unstained by Evans blue.

Discussion. Proteinuria occurs almost uniformly in normal laboratory rats. About half of this proteinuria is globulinuria. Proteinuria is a much less common finding in humans, but it does occasionally occur in the absence of demonstrable renal lesions.(11,12) An interesting parallel between rat and human proteinuria is the prevalence of tubular casts in the normal rat kidneys and the prevalence of tubular casts in the kidneys of humans known to have had gross proteinuria, whether frankly pathological or not.

The source if the urinary proteins, and the method of their passage into the urine, are two points of interest.

a. Source of the protein. The renal glomerulus has long been considered by many renal physiologists to be impermeable to plasma protein molecules. Such an attribute would make the capillaries of the glomerular tuft quite unique, as complete impermeability to protein cannot be ascribed to any other capillary bed. Thus, the human cerebrospinal fluid has a modicum of protein (20 to 65 mg %) with up to 6 mg of this as globulin. Warren(13) found the extracellular fluid of the human leg to contain 0.9% of protein. It is possible, of course, that the renal tubules secrete the urinary protein; but there has been no evidence adduced to this effect. That the protein in rat's urine is plasma protein is indicated by the passage of Evans blue into the rat's urine, as Evans blue is firmly and

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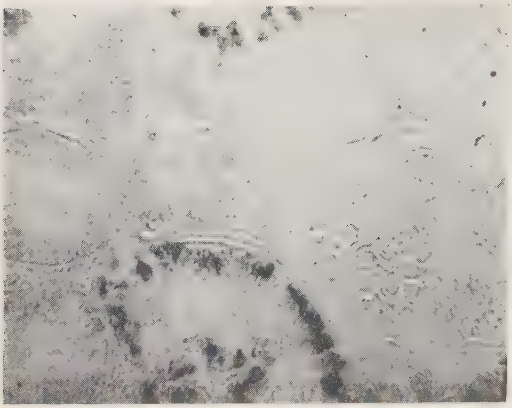

FIG. 1.
Kidney section of rat killed 24 hr after Evans blue injection (× 400). Glomerulus on right is unstained; some tubules are stained, others not.

preferentially bound to plasma protein. The data on Evans blue excretion must, however, be interpreted with reservations, as the protein-Evans blue complex represents an appreciable modification of the native plasma proteins, both as to molecular size and charge. Evans blue is a strongly acid substance and, as such, modifies the isoelectric point of the protein and might possibly modify the passage of the protein molecule across a membrane. There is also the possibility of a subtle effect of the Evans blue upon the kidney itself. However, no renal histopathology is noted after Evans blue injection.

b. Course of the protein from blood to urine. Evans blue appears earliest in the cytoplasm along the luminal borders of proximal tubule cells, and in intratubular casts, which are presumably protein in nature. Subsequently the dye pervades the cells and so approaches their basement membranes. This suggests that the tubules are absorbing the Evans blue, and, perhaps the protein with it, from the tubular lumen. If Evans blue did not pass into the tubular lumen by transfer across the tubular cells, the dye must have gained ingress by passing across the glomerular-capsular membrane, probably to be concentrated in the tubules into visible casts. Morphologically, the membrane appears to be non-secretory endothelium and would probably not disrupt the Evans blue-plasma protein complex. Also, the tubular casts were stained blue, so that the Evans blue-plasma protein complex probably remains intact at least until it reaches the lumen of the tubule. Whether the tubular cells selectively absorb the Evans blue, leaving the protein free in the tubule, cannot be ascertained on the basis of the present experimental evidence. However, should unmodified plasma protein cross the glomerular-capsular membrane in the same manner as the Evans blue-protein complex, it would seem likely, by analogy with other blood-borne substances, as glucose and sodium, which are believed to cross the membrane freely, that the protein would be at least partially reabsorbed by the tubules. And similarly, it would then seem likely that the Evans blue is absorbed in the tubules in association with plasma protein.

The absence of Evans blue stain from the parenchyma of the adrenal and the muscle cells of the myocardium is in sharp contrast to the staining of kidney tubules and liver parenchyma. The latter two tissues are believed involved in plasma protein transfer, and it is therefore unlikely that the stain of these tissues is a non-specific or a general protoplasmic reaction. That the liver and kidney react diversely with the protein complex is indicated by the different dispositions of dye within the parenchymal cells of the two organs. The Evans blue-stained particulate material within the tubular cells could be interpreted as either an aggregation of the Evans blue-protein complex, or as a staining of existing cellular structures by the Evans blue. In this connection, the similarity in form and size of the intracellular aggregates of dye to that described for mitochondria is suggestive. The total absence of Evans blue from some proximal tubules, and the heavy staining of other proximal tubules, even 24 hours after injection of the Evans blue, indicates variation in activity among the nephra themselves.

Summary. Proteinuria was found to be a usual occurrence in albino rats, with an average excretion during fasting of 3.0 mg of globulin and 3.3 mg of albumin in 24 hours. Marked variation in the daily urinary protein excretion was observed. Evidence is obtained indicating glomerular filtration and tubular reabsorption of an Evans blue-plasma protein complex, and, by inference, of normal plasma protein. Such tubular reabsorption possibly involves the mitochondria of the tubular cells.

Dr. E. F. Adolph gave invaluable suggestions, criticisms, and encouragement. Dr. James G. Wilson and Dr. Victor M. Emmel helped with the histological interpretations; their assistance is gratefully acknowledged.

Received November 1, 1949. P.S.E.B.M., 1949, v72.

Nutritional Value of Plant Materials. II. Prevention of Acute Uremia of the Newborn Rat by Vitamin B_{12}.* (17516)

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A very high incidence of mortality during the first 24-72 hours of the life of young rats born to mothers maintained on rations containing a commercial soybean protein preparation and DL-methionine as the only source of amino acids has recently been reported from this laboratory.(1) In about 50% of the litters cast, from one to all of the young develop a characteristic syndrome terminating in early death. At birth and for the first 24-36 hours these young, on visual inspection, appear to be in every respect normal, indistinguishable from those which survive and develop normally. They are strong and nursed by their mothers until the onset of the crisis. (The support of excellent lactation has been an outstanding feature of these rations). A rapid onset of dyspnea accompanied by cyanosis, weakness and emaciation is typical of the syndrome observed. Milk is always present in the stomachs of the young and very frequently one or two loops of the small intestine contain dark material which is visible through the abdominal wall. At this stage the blood urea as determined by the method of Archibald(2) reaches values of about 150-250 mg per 100 ml as compared to about 35 mg at birth and about 50-70 mg in normal appearing rats of the same age.(3) Death of these rats usually occurs within 4-6 hours after the first symptoms can be observed, although a few individuals have survived as long as 12 hours. Without inference concerning the nature of the biochemical or pathological lesion or the ultimate cause of death the syndrome is conveniently referred to as "acute uremia of the newborn."

Other investigators have reported early deaths of young rats(4-6) and mice(7) that had milk in their stomachs and they may have been dealing with the same syndrome. Zucker and Zucker(8) observed elevated concentrations of NPN and urea in the blood of rats of post weaning age which were fed rations devoid of animal protein. McGinnis *et al.*(9) refer to observation of an increased NPN concentration in the blood of chickens maintained on rations containing plant ingredients plus vitamin free casein. In this laboratory "acute uremia of the newborn" has never been observed on rations containing either alcohol extracted casein,(1) or a com-

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TABLE I.
Effect of Vitamin B_{12} on Acute Uremia of the Newborn.

	Inj. with 0.05 μg vit. B_{12}		Uninjected controls	
	From 13 uremic litters	From 13 normal litters	From 13 uremic litters	From 13 normal litters
Total No. of young	46	54	52	63
Young dead in 48 hr from all causes	7*	4	35	5
Young with acute uremia	1	0	36	0
	Normal appearing young		Uremic young	Normal appearing young
Blood urea mg/100 ml	50.2 ± 11.8 (18)†		207.3 ± 58.2 (9)†	53.7 ± 19.8 (18)†
21 day weaning wt, g	Males 35.4 ± 3.1† (30) Females 32.3 ± 3.7 (18)		Males 32.7 ± 5.4† (26) Females 32.5 ± 5.2 (17)	

Number of individuals given in parentheses.
* Includes 3 young killed by female.
† Standard deviation. Uremic litters refers to those in which one or more rats were observed in acute uremic crisis. The others are referred to as normal litters.

mercial soybean protein supplemented with liver extract (Wilson's 1:20) or condensed fish solubles. Since the ration used in these experiments is presumably low in vitamin B_{12} the experiments reported here were made. They appeared to be further justified by the preliminary observation that subcutaneous injection of an antipernicious anemia liver extract (Abbott) into the newborn rat prevented the development of "acute uremia of the newborn."

Experimental. The mothers of the young used for these experiments were fed the following ration: Commercial soybean protein (Archer Daniels Midland Co., Minneapolis) 274.4 g; DL-methionine, 5.6 g; salts 4(10) (Mn^{++} concentration reduced to 1/10th) 40 g; vegetable fat (Crisco) 70 g; corn oil, 10 g; sucrose, 610 g. Each kg of ration contained in addition thiaminechloride HCl, 5 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 5 mg; calcium pantothenate, 5 mg; cholinechloride, 1000 mg; i-inositol, 400 mg; para-aminobenzoic acid, 10 mg; nicotinic acid, 20 mg; 2-methyl-1,4-naphthoquinone, 5 mg; biotin, 0.2 mg; folic acid, 0.2 mg; d,l α-tocopherol, 100 mg; vitamin A (fish liver concentrate) 10,000 I.U.; vitamin D_3 (Delsterol) 1500 I.U. The mothers of the young used were F_2 or F_3 generation offspring from mothers on the same ration. They were kept on raised screens since weaning at 21 days of age except for a few days before parturition and during lactation. Within 2-3 hours after birth about half the number of live young in each litter were injected subcutaneously on the dorsal side with 0.1 ml of a solution containing 0.05 μg of crystalline vitamin B_{12} (Cobione Merck). Before withdrawal of the needle the site of injection was covered with a small piece of adhesive tape to prevent leakage. The injected young were identified by snipping off the tip of the tail.

Results and discussion. A comparison of the incidence of "acute uremia of the newborn" in injected and uninjected littermates is given in Table I. Only those individuals were counted as having acute uremia which were actually observed in the acute crisis. Litters in which no acute uremia was observed are tabulated as "normal" litters. A few deaths in each group occurred from crushing by the mothers, from unknown causes or probably from unobserved acute uremia in the uninjected control group. The fact that one injected individual was observed in acute crisis suggests that the quantity of vitamin B_{12} injected was not sufficient to prevent the syndrome in all cases. A typical example of the appearance of rats with the acute uremic

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10. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, v138, 459.

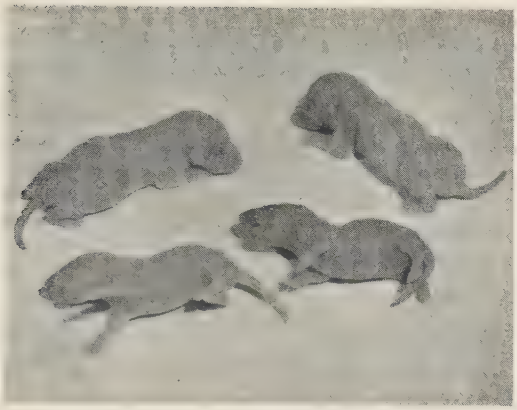

FIG. 1.

Forty-four hour old rats; the 2 rats in the upper portion of the figure were injected 2 hours after birth with 0.05 μg of vit. B_{12}; the 2 rats in the lower portion are their uninjected littermates. The appearance of the latter is typical for "acute uremia of the newborn." Blood urea of these injected rats was 76 and 40 mg per 100 ml; that of the uremic control rats 287 and 240 mg per 100 ml.

syndrome together with their injected littermates is shown in Fig. 1.

The urea concentration in the blood of a few of the rats was determined 40-48 hours after birth. Others were kept to determine weaning weights. The results included in Table I show that rats with "acute uremia of the newborn" have extremely high blood urea values of about 200 mg per 100 ml, whereas normal appearing uninjected young or those injected with 0.05 μg of vitamin B_{12} have a blood urea level of 50-60 mg per 100 ml. These levels fall into the range of values observed in a more extensive series studied in this laboratory(11) which revealed an early transient rise of urea in the blood of normal appearing young rats, even in those born to, and nursing, mothers maintained on the ration described here supplemented with 3% fish solubles or with 2% Wilson's liver extract 1:20. The present data suggest that either a single dose of 0.05 μg of vitamin B_{12} is not sufficient to maintain the blood urea in the range of values found at birth or at weaning or that a transient rise may occur regardless of a vitamin B_{12} supply. The weaning weights of the survivors were not significantly changed by the single injection of vitamin B_{12}.

The observations reported here do not explain the etiology of "acute uremia of the newborn" satisfactorily. Since the condition can be prevented by an early postnatal supply of vitamin B_{12} they suggest that the milk of the females maintained on the ration described here was low in vitamin B_{12} and that the fetal stores of this factor were likewise insufficient to overcome an early postnatal stress which may be associated with the adjustment of the newborn to the sudden supply of nutrients from the alimentary tract. The rapidity of its onset and its prevention by postnatal administration of vitamin B_{12} as well as the normal appearance of the young for the first 24 hours after birth further suggest that "acute uremia of the newborn" is of metabolic origin rather than a pathologic condition established *in utero*. Extensive data accumulated during experiments on reproduction of rats maintained on the ration described here show that often within a litter a few individuals show the acute syndrome while their littermates develop normally.(12) Furthermore, while a mother is maintained continuously on the same ration one litter may develop "acute uremia of the newborn" while the next litter does not, and vice versa. The incidence of the condition does not seem to increase greatly with successive generations. It appears therefore that under the conditions prevailing in these experiments circumstances of a marginal nature determine the incidence of the syndrome. Finally it has been observed(12) that a vitamin B_{12} deficient ration *per se* does not necessarily cause "acute uremia of the newborn." In view of these complex relationships clarification of the causes and of the nature of the syndrome must await further experimental evidence.

The acute uremia in newborn pigs reported by Madsen *et al.*(13) and the "toxemic-uremic syndrome in baby pigs fed on dried skimmilk" observed by Green *et al.*(14) may be related to the syndrome in rats described

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11. Halvorson, Harlyn O., and Schultze, M. O., unpublished.

12. Schultze, M. O., unpublished.

13. Madsen, L. L., Earle, I. P., Heemstra, L. C., and Miller, C. O., *Am. J. Vet. Res.*, 1944, v5, 263.

in this paper. In view of the high early mortality observed in swine the prophylactic use of vitamin B_{12} in these conditions should be investigated.

Summary. 1. Acute uremia of the newborn rat observed on rations in which a commercial soybean protein and DL-methionine furnish the only source of amino acids can be prevented by subcutaneous administration of 0.05 μg of vitamin B_{12} shortly after birth.

The assistance of Merck and Co., Abbott Laboratories, Wilson Laboratories, Lederle Laboratories, and DuPont and Co. with various supplies is gratefully acknowledged. Mr. Harlyn O. Halvorson kindly carried out the analyses for blood urea.

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Received November 1, 1949. P.S.E.B.M., 1949, v72.

The Volume Distribution and Equilibrium Time of Para-Aminohippurate.*
(17517)

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Sodium para-aminohippurate (PAH) is widely used for measurement of effective renal plasma flow and maximal tubular excretory capacity (Tm). Its volume distribution is of importance in studies dealing with rate of change in PAH plasma levels and excretion following single injections,(1-3) or with constant infusions before equilibrium has been established.(4,5) Information concerning volume distribution is also useful in calculating optimum priming doses for rapid achievement of the desired plasma level. The literature provides very few data on the PAH space. It has been stated to be 28% of body weight in the dog(6) and estimates of 4-5 liters(4) and 20 liters(5) have been made for man. We are therefore reporting its determination in man.

Methods. All patients were free of renal, cardiac, or other diseases which might be expected to alter fluid compartments. An intravenous priming dose of PAH was given over a 7-9 minute period and this was followed by a constant intravenous infusion for 1½-3 hours. In three patients the priming dose was omitted. Infusions (both priming and sustaining) were given at an extremely constant and accurately known rate by means of a motor driven worm screw pushing on the barrel of a 50 cc syringe. Urine was collected by catheter with bladder washing. The anticoagulant was heparin powder. Total PAH was determined according to Newman.(4) This method in our hands gives complete hydrolysis of acetylated PAH. Its use, in the cases reported resulted in no destruction of free PAH, as indicated by identical values in infusion solutions analyzed as free and total. The total amount of PAH excreted was subtracted from the total amount injected and this difference divided by the plasma level to arrive at the apparent volume distribution.

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* This work was supported by the Harrison Department of Surgical Research, the Department of Medicine, the William Pepper Laboratory, and the C. Mahlon Kline Fund for developments in the Department of Medicine.

† Harrison Fellow in Surgical Research.

‡ National Institute of Health Post-Doctorate Fellow.

§ Woodward Fellow.

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TABLE I.
Degrees of Equilibrium Attained with Various Durations of PAH Infusions without Priming Doses.

Patient PAH clearance PAH space	Time, min.	Plasma conc., mg %	Out/in ratio		
			Determined	Theoretical	Deter./theoret.
K.W.	83.2		.85	.86	.99
885 cc/min.	114	1.40	.90	.93	.97
37.1 L	143	1.45	.92	.97	.95
	175	1.47	.98	.98	1.00
J.D.	48.4		.82	.78	1.05
885 cc/min.	88.7	1.36	.87	.94	.93
28.0 L	112	1.44	.92	.97	.95
	141	1.46	.96	.99	.97
J.B.	37.1		.59	.63	.94
489 cc/min.	82.8	2.38	.89	.89	1.00
18.2 L	99.3	2.43	.88	.93	.95
	121	2.51	.95	.96	.99
	144	2.59	.95	.98	.97

As a criterion of equilibrium we have divided the rate of excretion in mg/min during a 15-20 minute period by the rate of injection in mg/min thus obtaining an "out/in ratio". As equilibrium is approached, this value approaches unity.

Results. Sixteen tests were done on 14 patients (all but 1 males, ages 11 to 50) and the volume distribution was calculated at 2 points in each test. The elapsed time at the first calculation point was 83 (68-121) minutes and at the second 130 (89-192) minutes. Most of the patients were excreting PAH in the urine at the same rate it was being injected (mean out/in ratio .96, S.D. .069, S.E. .017) by the end of the second period although not necessarily at the end of the first (mean .89, S.D. .094, S.E. .024) The mean apparent volume distribution at the end of the second period was 35.9% of body weight with a standard deviation of 7.3, a standard error of the mean of 1.8 and a range of 22.8 to 47.8. In terms of liters per square meter of body surface the mean was 13.4 with a standard deviation of 2.6, a standard error of the mean of 0.6, and a range of 9.1 to 17.7. All patients had priming doses except 3. Table I shows the time required in approaching equilibrium, in patients who received no primary dose. The percentage approach toward equilibrium is indicated by the out/in ratio (% of eventual plasma level is of course an identical concept). In Table I the figures for time intervals and plasma levels correspond to the mid point of the 15 to 30 minute urine collection period used in calculating the observed out/in ratios, whereas in space calculations figures corresponding to the end of the urine collection periods were used.

Discussion. The figure 36% of body weight lies between those reported for extra cellular fluid—15%(7) to 25%(8) and total body water—53%(7) to 70%,(9) indicating that PAH probably enters cells to some degree. It does not enter human red blood cells as several workers(10,11) have shown and we have confirmed. A recent statement(12) that human whole blood and plasma PAH concentrations are equal, was probably not intended and may have been an accident of wording. An alternative explanation to cell penetration might be that PAH reaches higher concentra-

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tions in some part of its volume distribution than in plasma. We have not considered in the present communication the question of expanding and shrinking apparent volume distributions. The mean space at the first calculation point was 34% and this rose to 36% by the time of the second calculation point (some 47 minutes later). This difference is statistically significant and could represent an expanding space. We are more inclined however to think that the finding arises from the fact that our priming dose in most cases was less than optimal and plasma equilibrium had not yet been achieved at the first calculation point. It is to be expected, of course, that any space calculation made before equilibrium has been achieved will be falsely low if the plasma level is rising and high if plasma level is falling. The magnitude of the volume distribution error is not known but it is probably small when the change in plasma level is occurring at a slow rate.

In practice of course, it is impossible to know what the eventual equilibrium plasma level will be and consequently one cannot detect the lack of equilibrium by comparing a specific plasma concentration with the eventual plasma level. It is known however what the eventual rate of excretion will be (*i.e.* equal to the rate of injection) and therefore examination of the out/in ratios is a far more useful index of equilibrium than judgments reached from comparison of several plasma determinations because, during a slow rise, the difference between successive samples may be no greater than the error of the method. For technical reasons equilibrium is desirable although not essential in all clearance determinations. If however, the rate of infusion is to be substituted for the rate of excretion,(5) then equilibrium is an absolute pre-requisite. Newman has suggested recently(4) that if the PAH prime be omitted entirely, equilibrium will be reached in 20-30 minutes. On the basis of out/in ratios however, our subjects did not seem to reach equilibrium under 2 hours even with a priming dose. It can be shown by substitution of our determined PAH space and reasonable clearance values for the average person in Newman's formula‖ that, when the priming dose is omitted, it should require 77 minutes to reach 90% of the eventual plasma level, 100 minutes to reach 95% and 153 minutes for 99%. Table I shows experimental values in close agreement with these calculations. Newman's estimate of 20-30 minutes equilibrium time was based on an erroneously low value of 4-5 liters for volume distribution.

We have not attempted to determine experimentally the exact size of the optimal priming dose for rapid achievement of equilibrium. It would appear from comparison of our data with and without priming doses that use of doses of 0.5 to 0.7 g injected over a period of 7-9 minutes does not appreciably hasten equilibrium over complete omission of a priming dose. It may be that use of a considerably larger dose would be of value. Berger, *et al.*(5) seem to have had some success with priming doses of 0.8 g, frequently reaching satisfactory equilibrium in 60-90 minutes.

Summary and conclusions. 1. The apparent volume distribution of PAH is 35.9 (S.D. ± 7.3) % of body weight.

2. If the priming dose is omitted and the plasma level allowed to rise slowly during an intravenous infusion of PAH, the plasma concentration appears to reach about 90% of its eventual level in 75 minutes, 95% in 100 minutes, and 99% in 150 minutes in the average patient, so long as the plasma concentration remains below that of beginning saturation of the tubular excretory mechanism.

‖ $P = \frac{IV}{C}(1 - e^{Ct/Vc})$ where P is the plasma concentration, IV is the infusion rate, C is the clearance, t is time and Vc is the apparent volume distribution.

Received November 1, 1949. P.S.E.B.M., 1949, v72.

A Rapid Bone Sectioning Technic.* (17518)

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This paper describes a recently developed apparatus and technic for rapidly cutting undecalcified bone sections sufficiently thin for microscopic and autoradiographic study. This technic is particularly applicable to the sectioning of old, dried bone or bone from mature animals. Sections may be cut at the rate of 20 to 40 per hour and they may be cut serially with the loss of not more than 150 microns of material between sections.

I. *The Sectioning Apparatus.* A conventional brain microtome was adapted by substituting a rotary, motor driven saw blade for the stationary knife blade. The bone to be sectioned was clamped in the reciprocating carriage which fed the bone to the saw. The

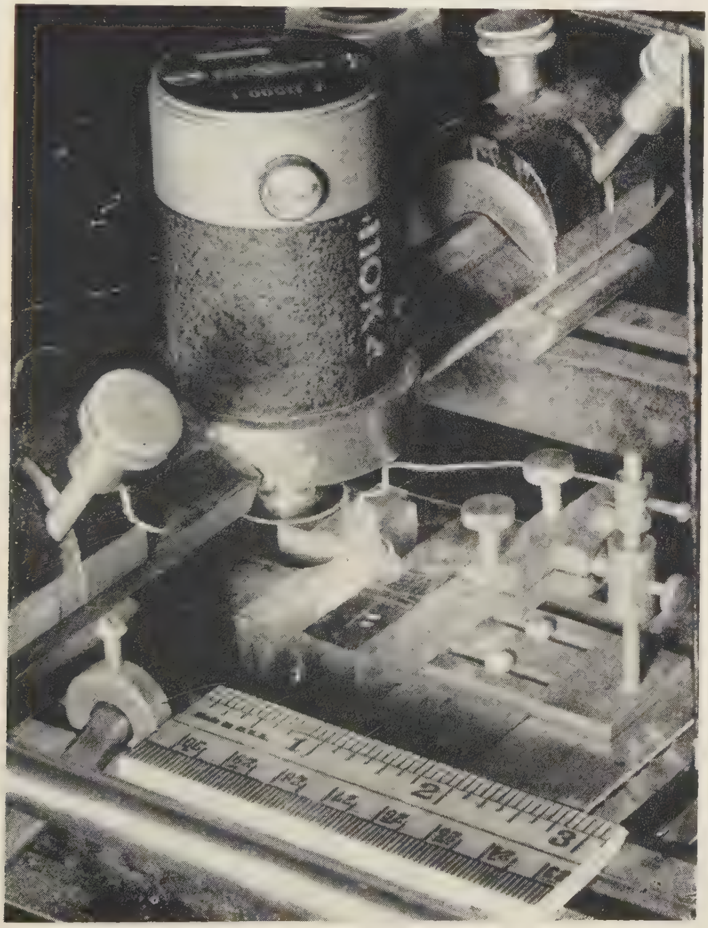

FIG. 1.
Bone-sectioning apparatus showing motor mounting and arrangement for clamping flexible plastic coverslip in contact with upper end of bone.

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* This work has been sponsored by the Office of Naval Research under Contract N6 ONR 260, T. O. 1.

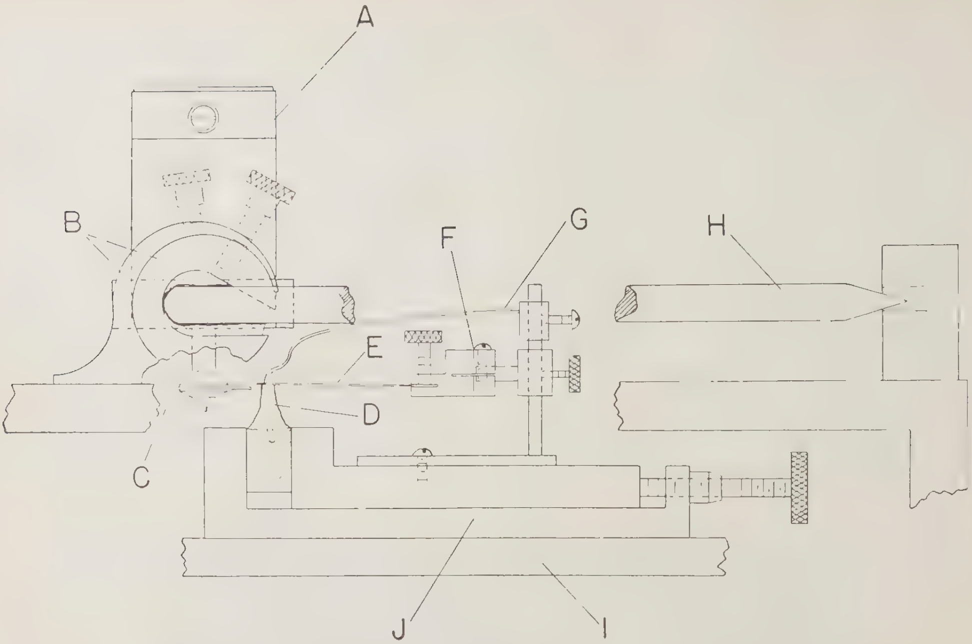

FIG. 2.

Details of bone-sectioning instrument. A. Motor. B. Motor mounting blocks which permit rotation of the motor about a horizontal axis perpendicular to the plane of the paper. C. Saw blade. D. Bone mounted in bioplastic. E. Flexible coverslip resting on upper surface of bone to retain cut section. F. Coverslip clamp. G. Wire for adjustment of pressure between coverslip and bone. H. Index arm for adjustment of attack angle of saw. I. Carriage way. J. Bone carriage.

motor used in this apparatus is a "war surplus" item† designed to drive a rotary switch in aircraft. Its small size (2½ inches x 1½ inches diameter) and the rigid ball bearing mounting of the armature are highly desirable features. However, other designs of motors would no doublt be usable provided the armature end-play was eliminated. The motor is mounted on a solid steel bar machined to fit the knife blade clamps on the chassis of the microtome. These clamps are adjustable so that rotation of the motor about an horizontal axis perpendicular to the path of the bone is possible. This permits a fine adjustment of the angle of attack of the saw blade, preventing it from cutting out of or into the bone as the section is cut. Figure 1 is a close-up photograph of the essential features of the apparatus. The circular saw blades‡ (commercially available for use in motor driven hand tools) are approximately ⅞ inch in diameter, have 88 teeth without set and are 150 microns thick. The blades are attached to the armature shaft, the end of which is machined to the diameter of the hole in the blade, by clamping it between thick, cone-shaped washers which serve to hold the blade rigid. The speed of the motor is controlled by a variable transformer.

Since these blades are manufactured in quantity by stamping, it is frequently found that the mounting hole is not exactly centered. This results in excessive vibration which interferes with the cutting of the very thin sections. This difficulty may be overcome by placing the blade in a mandrel which holds it over an accurately centered hole slightly larger than the original hole. The mounting hole is then ground to the slightly larger size of the centered hole. This, of course, necessitates the use of a machined motor shaft the same size as the enlarged hole.

II. *Preparation of Bones.* The cutting of very thin sections (20-30 microns) necessitates that the bone be surrounded with a shell

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† Bendix Radio, E. 11500-1, 28 Volts D.C.

‡ Dremel Manufacturing Co., 2400 Eighteenth St., Racine, Wisc.

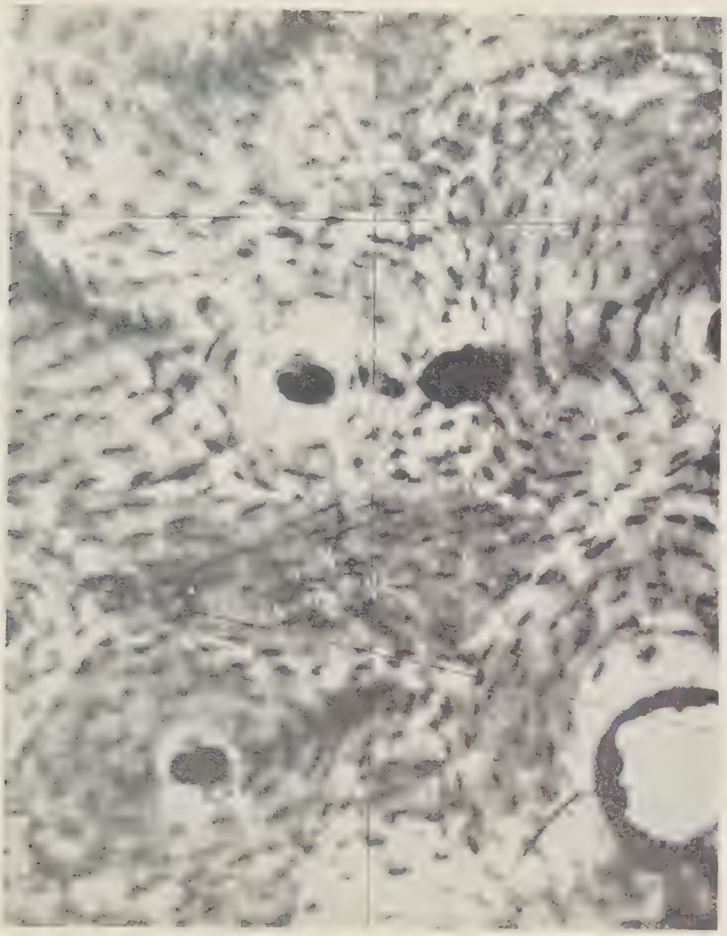

Fig. 3.
The cut section is approximately 30 microns thick and was cut from a piece of human bone from an individual 30 years of age at the time of death, which occurred 18 years ago.

of bioplastic which serves as a binder to prevent shattering of the section during cutting. The bone is prepared by embedding in Bioplastic§ which, after hardening, is trimmed to leave only a thin shell surrounding the bone. This is best accomplished by using a router bit in a motor driven hand tool. A rectangular block of the plastic is left attached to the end of the bone by means of which it may be clamped in the microtome carriage.

III. *Sectioning Procedure*. Before cutting each section, a flexible, plastic coverslip is placed over the free end of the bone. The coverslip is held in place by a holder mounted on a short metal rod, the base of which is attached to the carriage of the microtome.

A damper, consisting of a small piece of a microscope slide, is placed on top of that part of the coverslip immediately covering the free end of the bone. The damper, besides being attached to the coverslip holder by two strong threads, is held in place by a spring-wire keeper. The keeper is mounted in a position above the coverslip holder on the same rod. The end of the keeper is bent at right angles to the long axis and rests upon the damper. Fig. 2 is a schematic diagram of the arrangement of the various parts described. The heat generated by the saw blade softens the plastic coverslip just enough to secure the section after the bone and surrounding plastic are completely cut through. The coverslip also prevents the section from warping. The damper serves to reduce a slight vertical flutter which appears in the saw blades when traveling at high speeds and also to minimize other extraneous vibrations. The keeper facilitates a fine adjustment of the pressure exerted upon the coverslip. The operating speed of the motor, and hence the saw blade speed, depends upon the type of bone being cut (degrees of hardness, inherent structure, etc.); the cross sectional area of the bone; the speed at which the bone is fed to the saw blade; and the thinness of the desired sections. Due to the extreme variability of these factors, it is difficult to set down definite rules for governing the saw blade speed; hence an insight into the problem must be acquired largely through experience.

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§ Ward's Natural Science Establishment, Rochester, N.Y.

The cut should be made in one firm smooth motion as rapidly as possible so that the section will be in contact with the saw blade a minimum period of time. It has been found that the bone should be moved against the saw blade at a speed approximately 0.5 cm/sec. for best results. After cutting, the sections can be removed from the coverslip by means of a thin razor blade.

If sections are being cut from a bone having a relatively small cross sectional area, a section will sometimes be lost because not enough of the coverslip is softened to secure the section. This loss can be prevented by stopping the cut before the trailing edge of the plastic is completely severed. The cut can then be finished with a thin razor blade without loss of the section. Figure 3 is a photomicrograph of a section of human bone cut by this method. The cut section is approximately 30 microns thick and was cut from a piece of human bone from an individual 30 years of age at the time of death, which occurred eighteen years ago. The bones were kept in a sealed glass jar in the dry condition for the eighteen-year period. They were prepared for sectioning by embedding in Bioplastic as described above without any other treatment of any kind. Several thousand sections have been cut by this method.

Received November 1, 1949. P.S.E.B.M., 1949, v72.

Effect of Pilocarpine on Epinephrine Reversal Produced by 2-(N-p'toly-N-(m'-hydroxyphenyl)-aminomethyl)-Imidazoline. HCl (C-7337)* (17519)

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The recent introduction of a number of new drugs, which antagonize the pressor action of epinephrine, has created renewed interest in the well known phenomenon of epinephrine reversal. This problem is of considerable practical importance since many of these newer agents are being used in the treatment of human disease as a possible substitute for surgical sympathectomy. There have been a number of hypotheses suggested which attempt to explain the phenomenon of epinephrine reversal, none of which seems adequate. Substances which produce an epinephrine reversal are considered to block some part of the neuro-muscular apparatus which is concerned with the excitor actions of epinephrine, thus uncovering the inhibitory functions of epinephrine. The antagonism of epinephrine reversal by a wide variety of agents has been reported in recent months.(1-3)

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* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

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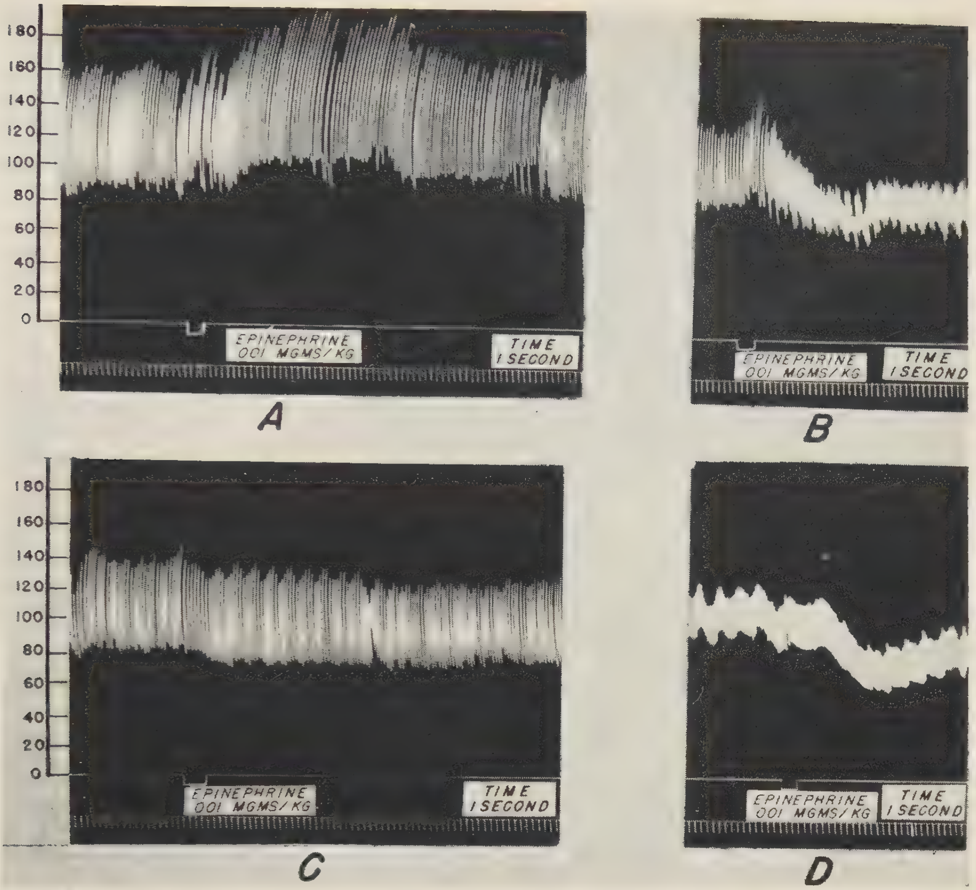

FIG. 1.

Unanesthetized dog. Events reading in sequence from left to right. Blood pressure recorded from femoral artery. A—Control injection of epinephrine. B—Epinephrine after 4.0 mg per kilo of C-7337. C—Epinephrine after C-7337 and 0.25 mg per kilo of pilocarpine. D—Epinephrine after C-7337, pilocarpine and 10 minutes after 1.0 mg per kilo of atropine.

Although there has been little similarity in the chemical or physiologic action of most of these agents, there has been some agreement that pilocarpine and other parasympathomimetic agents were most active. Since this phenomenon seems to us to have an important bearing on our concepts of autonomic physiology and pharmacology we have quantitated this effect using a new adrenolytic agent C-7337.(4)

Methods. Unanesthetized dogs were tied to an operating table and under procaine local anesthesia, a femoral artery and vein were exposed and cannulated. The artery was connected to an Anderson glass capsule manometer(5) for recording of arterial pressure and pulse. The vein was connected to a system which permitted easy injection of drugs. All drugs were given intravenously and washed in with 10 ml of normal saline. Each animal was heparinized by the injection of 1 mg/kg of heparin and the manometer system was filled with heparinized saline. Recordings were made on smoked kymograph paper. Epi-

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nephrine was injected in a dose which produced approximately 40 mm rise in pressure, following which 4 mg per kg of C-7337† was injected. This dose of adrenolytic agent is approximately 4 times that required to block the action of epinephrine(4,6) and produces effects which last for at least several hours. Following the injection of the adrenolytic agent, epinephrine was injected again in the same dosage as used in the control period. This invariably produced a fall rather than a rise in blood pressure. Gradually increasing doses of a variety of substances were then injected to test their effect on the epinephrine reversal. Dosages were continued until the epinephrine reversal was abolished or toxic symptoms were produced. Because of the fact that the injection of epinephrine after an adrenolytic agent produces an effect on the circulation comparable to that of isopropyl norepinephrine, it seemed of interest to determine the effect of pilocarpine on the depressor action of the latter compound.

Results. We have found that pilocarpine, physostigmine, and choline all possess the ability of abolishing epinephrine reversal. Kymograph records from a typical experiment are illustrated in Fig. 1. Since pilocarpine produced the most striking results, this agent was studied in sufficient detail to describe the relationship between dosage and effect. A total of 5 dogs were used and the results of these studies are summarized in Fig. 2.

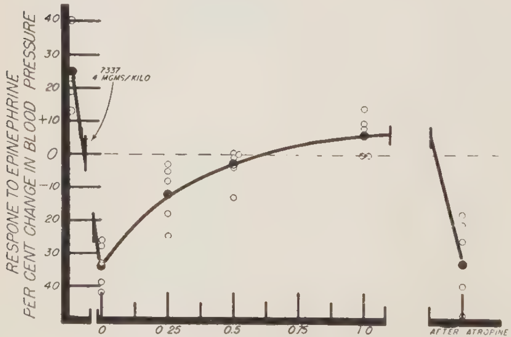

FIG. 2.

Summary of 5 experiments in unanesthetized dogs showing the relationship between dosage of pilocarpine and antagonism of epinephrine reversal by C-7337.

Following the abolition of epinephrine reversal, atropine in a dosage of 1 mg/kg was injected intravenously. Epinephrine injected within 5 minutes after atropine produced only slight effect upon blood pressure. If, however 10 minutes were allowed to elapse before epinephrine was reinjected, it invariably produced the same depressor effect which is observed immediately following administration of the adrenolytic agent.

In 6 experiments, using isopropyl norepinephrine, it was found that pilocarpine in a dosage of 0.5 mg/kg blocked the action of .001 to .004 mg/kg of isopropyl norepinephrine.‡ Fig. 3 illustrates one of these experiments. Of interest also was confirmation of the observations of Raymond-Hamet(7) that pilocarpine antagonizes the action of acetylcholine as shown in Fig. 4.

Further evidence of the similarity of action of isopropyl norepinephrine and the inhibitory effects of epinephrine following an adrenergic agent was shown by the fact that the pilocarpine blockading action could be reversed by atropine. Similar results have been reported by Fromherz.(8)

That antagonism of C-7337-epinephrine reversal by pilocarpine was not limited to this one adrenolytic agent was obtained from experiments showing that dibenamine-epinephrine reversal was also blocked by pilocarpine.

Discussion. The results presented above are difficult to explain in terms of present concepts of function of peripheral autonomic mechanisms. The similarity of action of isopropyl norepinephrine and the cardiovascular inhibitory actions of epinephrine after an adrenolytic agent suggests a common mode of action. The effect of parasympathetic stimulants and depressants on these phenomena suggest some relationship between the receptive mechanisms of the inhibitory sympathetic end organs and the receptive substance of the

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† Generously supplied by Ciba Pharmaceutical Products, Inc.,

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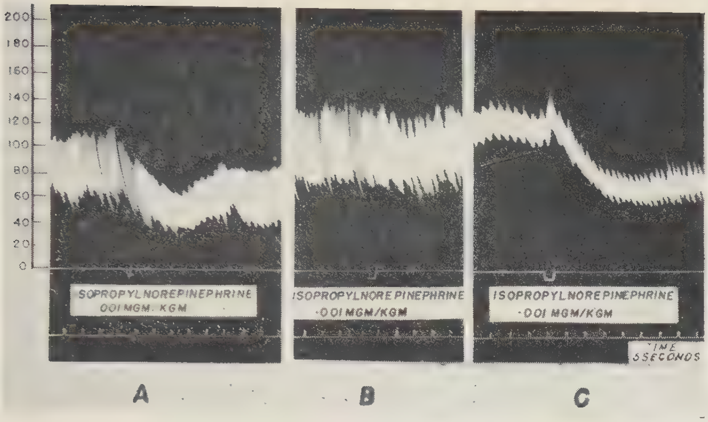

FIG. 3.
Unanesthetized dog. Events leading in sequence from left to right. Blood pressure recorded from femoral artery. A—Control injection of isopropyl norepinephrine. B—Isopropyl norepinephrine after 0.5 mg per kilo of pilocarpine. C—Isopropyl norepinephrine after pilocarpine and after 1.0 mg per kilo of atropine.

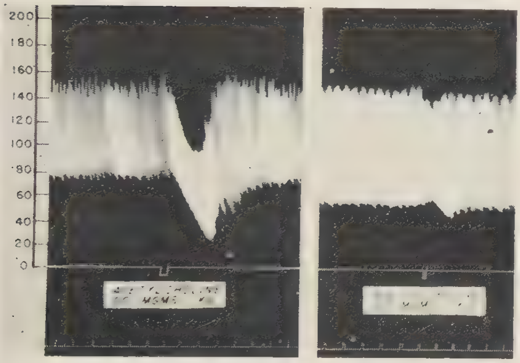

FIG. 4.
Unanesthetized dog. Blood pressure recorded from femoral artery. Left—Control injection of acetylcholine. Right—Same dose of acetylcholine 10 minutes after 1 mg per kilo pilocarpine.

parasympathetic system. This relationship, however, is not immediately clear, but points to the need for a more complete study of the interaction of autonomic drugs.

Conclusion 1) Epinephrine reversal produced by 2-(N-p′tolyl-N-(m′-hydroxy-phenyl)-aminoethyl)-Imidazoline HCl (C7337) may be abolished by the injection of pilocarpine and other parasympathomimetic agents.

2) The reversing action of such an adrenolytic agent may be reinstated by injection of atropine.

3) Pilocarpine is also effective in decreasing the depressor action of isopropyl norepinephrine and of acetylcholine.

Received November 1, 1949. P.S.E.B.M., 1949, v72.

Chromatographic Demonstration of Histamine Release in Anaphylactic and Trypsin Shock. (17520)

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A large body of evidence has accumulated to indicate that histamine is released in anaphylactic shock and similar reactions in amounts adequate to account for many features of these phenomena.(1-4) The methods employed to ascertain the presence of histamine in such phenomena were based mostly on the biological activity of this substance. Convincing chemical proof has been lacking since adequately sensitive and specific chemical methods to detect small amounts of histamine in the presence of other substances have been developed only very recently.(5,6) A preliminary report has described the identification of histamine released from sensitized rabbit blood cells *in vitro* by paper chromatography(7) and further proof for the specificity of the method has been presented.(8) The purpose of this communication is to present more complete details as to the method and to report the results obtained by its use in anaphylactic and trypsin shock in dogs.

Method. Blood samples (40 ml) were collected in heparinized or oxalated tubes from dogs undergoing shock. The samples were centrifuged and the plasma (approx. 20 ml) separated. Each plasma sample was diluted with an equal volume of distilled water in a shell vial and 3 g of a salt mixture (consisting of 62.5 g anhydrous Na_2SO_4 and 10 g $Na_3PO_4 \cdot H_2O$) was added for each 10 ml of the diluted plasma, according to the procedure of McIntire.(9) To each 20 ml of diluted plasma 10 ml of butanol were added to extract the histamine and the samples were shaken in a mechanical shaker for 30 minutes. The aqueous, protein and butanol layers were separated by centrifugation. The butanol layer was drawn off and placed into a clean shell vial. The butanol extraction was repeated and all butanol extracts from each plasma sample were combined. (An increase in the amount of butanol was not found to improve the histamine extraction significantly, with amounts of plasma up to 20 ml. Increasing the number of butanol extractions, similarly, had no appreciable effect on the recovery of histamine when added to control plasma.)

To the combined butanol extracts were added 5 ml of 0.5 N H_2SO_4. The tube was shaken for 10 minutes, centrifuged briefly and the aqueous bottom layer transferred to a small glass-stoppered vial by means of a suction-bulb pipette.(9) The butanol solution was again extracted with 2 ml of H_2O in a similar manner and this second aqueous layer combined with the acid.

The combined H_2SO_4 and H_2O extracts were neutralized with 10% NaOH in a small glass-stoppered vial and 1.5 g of the previously mentioned salt mixture and 2.5 ml of butanol were added. After shaking the vial for 20 minutes, the layers were separated by brief centrifugation and the butanol layer transferred with the suction-bulb pipette to an applicator pipette in contact with the filter paper strip* which was stretched horizontally

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* The filter paper used in this and previous work was supplied by the Eaton-Dikeman Co., Mount Holly Springs, Pa., in convenient rolls, $\frac{5}{16}$″ wide.

TABLE I.
Histamine Content (μg Base per 20 ml) of Dog Plasma Before and During Anaphylactic Shock.

Dog. No.	Degree of shock	By paper chromatography Before	By paper chromatography During	By bioassay Before	By bioassay During
1	Fatal	0	>10	<1	50
2	,,	0	>10	<1	48
3	,,	0	>10	<1	16
4	Mild	0	0	<1	< 1
5	Fatal	0	>10	<1	28

TABLE II.
Histamine Content (μg Base per ml) of Dog Plasma Before and During Trypsin Shock.

Dog No.	Dose trypsin, mg/kg	Degree of shock	By paper chromatography Before	By paper chromatography During	By bioassay Before	By bioassay During
1	50	Mild	0	>3<5	Not done	
2	100	Fatal	0	>10	<1	38
3	100	,,	0	0	<1	<1
4	100	,,	0	>10	Not done	
5	100	,,	0	>10	,,	,,
6	100	,,	0	>10	,,	,,

over a hot plate (temperature at level of strips 70-70°) as previously described.(10) The extraction of the aqueous layer was repeated with 1 ml of butanol which was added to the first butanol extract in the pipette.

After application of the final butanol extract to the paper strip, the strips from both control and shock samples of blood were stapled to narrow cardboard at intervals of ½ cm. Chromatography with butanol saturated with 10% NH_4OH, and color development with diazotized p-bromoaniline was carried out as described in a previous communication.(8) Histamine, when present in amounts exceeding 2-3 μg, made its appearance on the strips as a red band at an R_f value of approximately 0.56. Control strips to which known amounts of histamine had been applied were run simultaneously. This permitted a rough approximation of the amount of histamine in the 20 cc plasma samples.

Anaphylactic shock. Blood samples were collected from 5 dogs sensitized to horse serum both before and 1-3 minutes after i.v. injection of the antigen. The release of histamine as shown by chromatography and bioassay[†] is summarized in Table I. One of the experiments is illustrated in Fig. 1.

Trypsin shock. Six dogs were injected i.v. with crystalline trypsin (Armour). Blood samples were taken before and during shock. The results are summarized in Table II. One of the experiments is illustrated in Fig. 2.

Release of histamine in dogs shocked with peptone could not be demonstrated by chromatography since the amounts of peptone required to produce severe or fatal shock (1 cc of 20% Bacto-Protone (Difco)/kg) interfered markedly with the chromatographic procedures. This was in part due to the interference of peptone with proper separations on the chromatograms, as well as to the presence of substances in the peptone solutions which give a strong color reaction with the diazotized p-bromoaniline used as the color reagent on the chromatograms. Attempts to separate these interfering substances by either electrodialysis or repeated butanol extractions of peptone solutions resulted in fractions which did not produce shock in the dogs. Addition of small amounts of peptone solutions to normal rabbit blood, however, caused release of histamine into the plasma which could be demonstrated by the chromatographic procedure.

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[†] All bioassays were kindly performed by Dr. Lorenzo Giscafré and Dr. Wilson T. Beraldo.

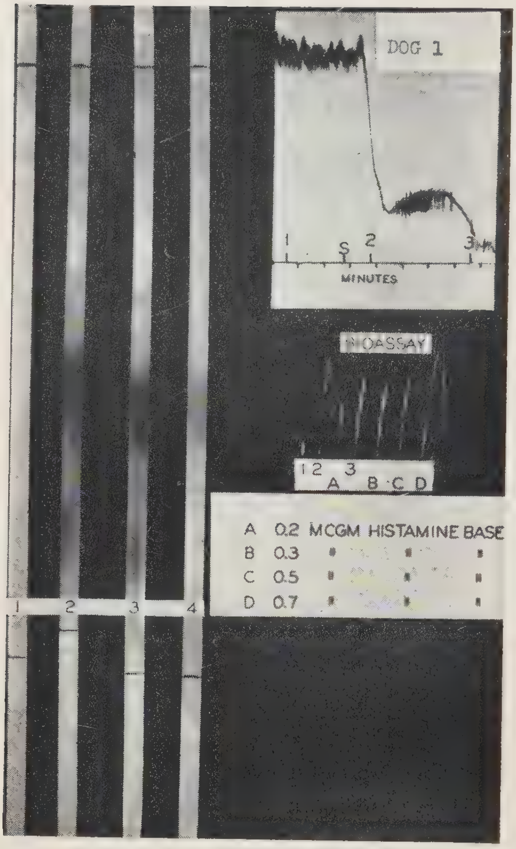

FIG. 1.

Paper chromatograms, blood pressure record and bioassay from a dog during anaphylactic shock.

1, 2 and 3 on B.P. record signify blood samples drawn S—antigen injection.

1, 2 and 3 on chromatograms and bioassay record correspond to the respective blood samples.

4 on chromatograms represents 10 μg pure histamine base chromatographed as control. Note the much heavier bands of histamine on strips 2 and 3 at the same location and absence on strip 1. The substance(s) on strip 2 and 3 responsible for the dark bands below the histamine bands have not been identified.

A, B, C, D on bioassay record represent standard amounts of histamine added to the muscle bath for calculation of the amounts of histamine in the plasma samples.

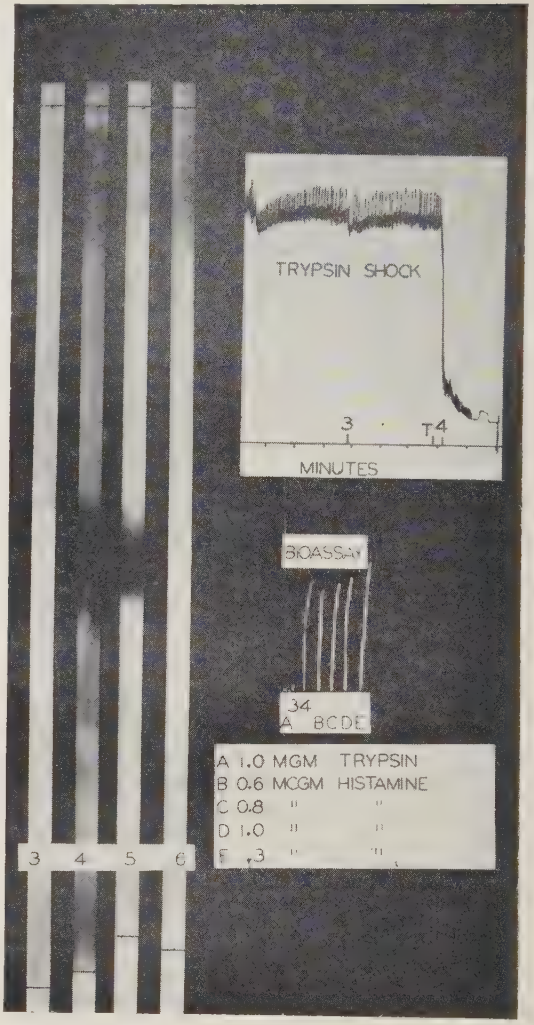

FIG. 2.

Paper chromatograms, blood pressure record and bioassay from a dog during trypsin shock.

3 and 4 on B.P. record signify blood samples drawn T—trypsin (100 mg/kg) injection.

3 and 4 on chromatograms and bioassay record correspond to the respective blood samples.

5 on chromatograms represents 10 μg pure histamine chromatographed as control.

6 on chromatograms represents 40 mg trypsin chromatographed as control.

A on bioassay record represents a trypsin control.

B, C, D, E on bioassay record represent standard amounts of histamine added to the muscle bath for calculation of the amount of histamine in the plasma samples.

Discussion. The experiments described identify histamine liberated during anaphylactic and trypsin shock in dogs. While the chromatographic method as employed routinely in this study does not permit exact quantitation, a better estimation of the amount of histamine can be achieved by chromatographing a series of control strips with graded amounts of histamine along with aliquots of the sample. For example—aliquots of the first butanol extract of the shock plasma obtained from one of the dogs were chromatographed with a standard series of histamine samples. A butanol sample containing 7.8 μg histamine (calculated on the basis of bioassay of the plasma) showed a chromatogram falling between standard chromatograms of 5 and 10 μg histamine and corresponding most closely to the 7 μg standard as judged by an unbiased observer. The chromatographic method with a limit of sensitivity of 2-3 μg per sample is obviously inferior to bioassay in this respect but is superior so far as specificity is concerned. The demonstration of histamine release in amounts roughly equivalent to those observed in the anaphylactic experiments in a high percentage of the trypsin shock experiments confirms an earlier report from this laboratory(3) but is in contrast to a more recent report, also from this laboratory.(11) We are not at present prepared to explain the discrepancy.

Summary. A method for the chemical identification of histamine in blood by paper chromatography is described in detail and experiments are presented to show the release of relatively large amounts of histamine in dogs undergoing anaphylactic and trypsin shock. Peptone, when injected into dogs in amounts sufficient to produce severe shock, interfered with the identification of histamine which could, however, be found on the chromatograms after addition of small amounts of peptone to rabbit blood (*in vitro*).

The author wishes to express his appreciation to Dr. Carl A. Dragstedt for many helpful suggestions.

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Received November 1, 1949. P.S.E.B.M., 1949, v72.

Inhibition of Hyaluronidase by Aromatic Compounds. (17521)

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The aim of our experiments was to find substances inhibiting the action of hyaluronidase. Repeating Guerra's work,(1) we(2) confirmed his observations that this enzyme was inhibited by sodium salicylate. Prior to this, hyaluronidase was found to be inhibited by heparin, gastric mucin, pseudo-globulin,(3) morphine,(4) hyaluronic acid,(5) estrogens(6) and, of course, enzyme inactivators. These experiments were carried out *in vivo*. By studying the effect on the area of spread in the skin, it was found that ascorbic acid,(3) glycerin,(7) triacetin,(7) arsenious oxide,(7) lecithin,(8) peptones,(8) urethane, sodium diazobenzene sulfonate,(9) hirudin,(7) human urine(10) and diazotized proteins(11) enhance this enzymatic action. Obviously, these lists fail to reveal chemical structures which are essential for anti-hyaluronidase activity. We, therefore, undertook to study a series of simple chemically related compounds and used the turbidimetric method described by Kass and Seastone.(12)

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It is based on the observation that pure hyaluronate in a buffered solution produces a fairly stable colloidal suspension when mixed with diluted acidified horse serum. There is a reduction in turbidity when hyaluronate is depolymerized by hyaluronidase. The efficiency of various inhibitors can be studied by measuring the turbidity of the various mixtures when compared to controls. This turbidity is a function of the undecomposed hyaluronic acid present. When the enzyme is introduced into the system, the turbidity produced holds an inverse relationship to the hyaluronidase content.

To determine the effect of a particular chemical, a series of freshly prepared dilutions of the enzyme were prepared. Five tubes were used with serial dilutions running in the order of 10, 20, 30, 40 and 50 γ of enzyme per cc. Since the concentration of the original solution was 100 γ per cc, 0.1, 0.2, etc. of this solution was placed in the tube and diluted to 0.5 cc with buffer pH 6.0 to maintain constant volumes. To each of these dilutions, 0.5 cc of the hyaluronate solution (0.04 mg/cc) was then added. Immediately after this, 0.1 cc of the compound to be tested was added to another tube which contained 50 γ of hyaluronidase and the substrate. All tubes were then incubated for 30 minutes in a constant temperature water bath at 37.5°C. The remainder of the procedure was performed as outlined by Kass and Seastone. Whenever possible, several determinations were made the same day. Each day a curve was plotted anew. This was desirable because of the variability and colloidal instability of proteins in the diluted horse serum which served as an indicator for undecomposed hyaluronic acid present. All the curves were S shaped in form and were prepared by using the dilution as abscissa and the turbidity, in terms of the reading on the Klett-Summerson photo-electric colorimeter, as ordinate. Red filter No. 66 was used.

As an example, the curve obtained using sodium salicylate is illustrated in Fig. 1. Here the reading on the colorimeter corresponding to 50 γ of hyaluronidase is 35. When 10 mg of sodium salicylate was added, the reading was 47, which corresponds to a total quantity of 34.7 γ of enzyme. This difference must have been inactivated by the sodium salicylate added. From the concentration and volume of the inhibitors, the amount of enzyme inactivated by 1 mg can be calculated. This procedure was repeated for each one of the various inhibitors tested.

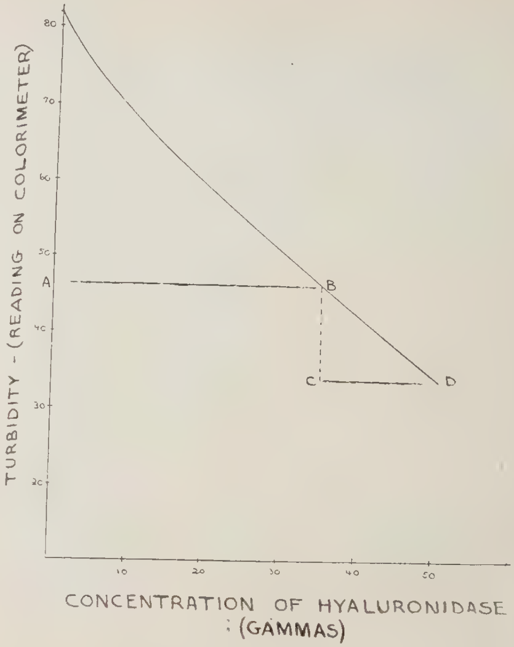

Fig. 1.

Method used to calculate activity of inhibitors.

D—Turbidity equivalent to 50 γ of hyaluronidase.

B—Turbidity obtained by the addition of sodium salicylate.

AB—Quantity of hyaluronidase remaining after using sodium salicylate.

CD—Quantity of hyaluronidase inactivated by 10 γ of sodium salicylate.

Before any inhibitor was used, certain criteria were established to eliminate invalidating factors. These were as follows:

1. Solutions of the inhibitor must be clear.

2. Alcohol solutions were diluted 1:10 with buffer pH 6.0 to ascertain whether mere dilution produced a precipitate.

3. Non-specific protein precipitating action of the inhibitors were tested by adding them to the acidified normal horse serum which was diluted with 3 parts of buffer pH 4.2.

4. Extensive studies of all pHs were carried out (Table I). Two buffers of pH 6.0 and 4.2 were checked periodically on a Beckman

TABLE I.
Comparative List of Hyaluronidase Inhibitors.

%	Inhibitor		pH of buffer (pH 6.0) + inhibitor	pH of mixture: buffer pH 6.0 + buffer pH 4.2 + inhibitor	γ of hyaluronidase inactivated by 1 mg of inhibitor
1	m-hydroxybenzoic acid	alc	5.52	4.25	3.2
1	p-hydroxybenzoic acid	"	5.65	4.25	3.6
1	ethyl p-hydroxybenzoate	"	6.12	4.26	7.0
5	phenol	aq	6.02	4.23	7.5
5	resorcinol	alc	6.12	4.25	8.4
1	o-nitrophenol	"	6.06	4.25	19.7
1	guaiacol	aq	6.02	4.24	24.0
1	m-cresol	"	6.03	4.26	25.0
1	methyl p-hydroxybenzoate	alc	6.12	4.25	28.0
1	propyl p-hydroxybenzoate	"	6.12	4.25	31.0
1	4-aminosalicylic acid	"	5.68	4.26	31.7
1	o-cresol	"	6.12	4.26	37.5
1	dinitrophenol	"	5.72	4.25	39.6
1	p-chlorophenol	"	6.12	4.25	42.0
1	o-chlorophenol	"	6.13	4.25	44.2
1	picric acid	aq	5.68	4.26	46.4
1	butyl p-hydroxybenzoate	alc	6.12	4.25	93.0
0.1	hexylresorcinol	"	6.12	4.26	400.0
.025	diamyl phenol	"	6.12	4.27	600.0
.052	2,5 ditertiary butylhydroquinone	"	6.10	4.27	669.3
10	sodium salicylate	aq	6.02	4.26	1.5
5	sodium gentisate	"	5.85	4.27	0.7

pH meter. The readings were between 5.95 and 6.02 for the former and 4.15 and 4.20 for the latter.

To ascertain the pH of the solution containing buffer pH 6.0 and the same proportion of inhibitor as in the studies, *i.e.* 1:0.1, a thorough investigation was undertaken with each inhibitor (Table I) and the values obtained varied between 5.52 and 6.13. It is important also to note that there is no direct relationship between the variations in pH and the potency of drug inhibition and that the activity of hyaluronidase is maximal within these two extremes.

When the two buffers (6.0 and 4.2) were mixed in the same proportion as in the determinations, *i.e.* 1:3, the average pH value of the mixture was 4.25 ± 0.02. The effect of each inhibitor on this value was then studied by adding 1:10 of the inhibitor to the mixed buffers. As can be observed, the values are between 4.22 and 4.27 and well within the limits of experimental error under the conditions of this experiment. Thus, the influence of the inhibitor to displace the pH of the buffering system was eliminated and the action on the acidified horse serum was a variable of the enzyme factor alone; non-specific action of the inhibitors was eliminated.

All data are tabulated in terms of gammas of hyaluronidase inactivated by one milligram of each compound. Since ethyl alcohol (95%), in the volume used, had no effect on the production of turbidity, it was used as a solvent for most of the drugs. In all other cases aqueous solutions were used.

Discussion. At this time we may speculate on the clinical importance of these findings. If an etiological relationship can be presupposed between hyaluronidase and acute rheumatic fever, the need for massive doses of sodium salicylate should be explained by our findings, since this drug is a weak inhibitor of hyaluronidase. As a matter of fact, Coburn(14,15) advocated plasma levels of at least 25 mg per 100 cc to secure prompt and progressive subsidence of the inflammation. This concentration usually makes the patient toxic with tinnitus, nausea, etc. Recently, Fulton *et al.*(16) reported that concentrations of sodium salicylate up to 100 mg % failed to inhibit hyaluronidase *in*

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vitro. In order to obtain only a slight effect of this enzyme in our experiments, we were forced to raise the salicylate concentration ten times higher than Fulton by using 10 mg of sodium salicylate per cc which is beyond the range of therapeutic dosage. In view of recent clinical investigations,(17) there is a possibility that the modus operandi of salicylates is a stimulation of the adrenal glands liberating a hormone which relieves the rheumatic manifestations. Sodium gentisate, which is a metabolic product of salicylate is even weaker. In the study of 4-amino salicylic acid (PAS), the anti-hyaluronidase activity approaches that of ortho cresol with, however, a higher therapeutic index.

Of the utmost importance is the fact that neither benzoic acid nor para aminobenzoic acid inhibit hyaluronidase, whereas their hydroxyl derivatives do.

Summary. The anti-hyaluronidase activity of phenol substitution products increases markedly with the length of the carbonic side chain. Salts of aromatic acids, like sodium salicylate, have an activity of a lower order as compared with that of phenols with long carbonic side chain. A moderate increase in activity is also obtained by substituting chlorine, nitro or amino groups.

As the data indicate, there exists in general, a parallel course of the inhibitory power of the substances and their phenol coefficient.

We wish to thank S. Joffe for valuable laboratory assistance.

Both the enzyme and substrate used in these experiments were obtained from the Schering Corporation.

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Received November 1, 1949. P.S.E.B.M., 1949, v72.

Synergistic Action of Estrogenic Hormone and X-rays in Inducing Thymic Lymphosarcoma of Mice.* (17522)

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Lymphocytic neoplastic disease was induced by estrogens(1) or x-rays(2) in mice of certain genetic constitution when these agents were given in appropriate doses. The carcinogenic hydrocarbons are another group of lymphoma-inducing agents.(3) Synergistic or additive lymphomogenic effects of x-rays and the carcinogenic hydrocarbon, methylcholanthrene, were demonstrable(4) providing lymphoid tissue of the test animals was susceptible to the neoplasia-inciting action of each of the agents used independently.(5) In the present experiment the synergistic action of x-rays and estrogens was investigated. It had previously been demonstrated that the CBA and Bagg albino stocks of mice are susceptible to the induction of lymphomas by the action of either estrogens or x-rays.(6) A single dose of 200 r of x-rays is a minimum inducing dose; weekly injections of 5 micrograms of estradiol dipropionate for 14 weeks

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* This investigation has been aided by grants from the National Cancer Institute, the Jane Coffin Childs Memorial Fund for Medical Research, and the Cancer Fund of the Graduate School of the University of Minnesota.

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TABLE I.
Occurrence of Lymphomatosis in Bagg Albino Mice Receiving Threshold Doses of Lymphomogenic Agents Either Singly or in Combination.

Agent	Total mice	Mice over 250 days	Total lymphoma	Mediastinal lymphoma	Age lymphoma (in days)	Dead and negative	No. living*
200r†	105	70	3	1	365	10	92
EDP‡	59	30	2	2	350	7	50
200r + EDP	70	36	15	12	283	13	42
Controls	101	101	32	1	725	69	0
18MC§	108	76	1	0	490	23	84
200r + 18MC	109	75	3	2	365	18	89
EDP + 18MC	58	19	3	1	285	5	50

* The oldest EDP-treated test animals (alone or in combination) are 500 days of age. The oldest MC-treated mice are 700 days of age.

† 20 r of x-rays in one exposure.

‡ Estradiol dipropionate—5 μg weekly for 14 weeks. This estrogen was supplied by Ciba Pharmaceutical Products through the courtesy of F. E. Houghton.

§ Methylcholanthrene—18 skin paintings with 0.25% solution in benzene.

TABLE II.
Occurrence of Lymphomatosis in CBA Mice Receiving Threshold Doses of Lymphomogenic Agents Either Singly or in Combination.

Agent	Total mice	Mice over 250 days	Total lymphoma	Mediastinal lymphoma	Age lymphoma (in days)	Dead and negative	No. living*
200r	148	83	6	1	410	8	134
EDP	57	42	2	1	294	2	53
200r + EDP	55	33	11	11	293	2	42
Controls	63	63	20	0	608	43	0
18MC	126	103	1	0	405	20	105
200r + 18MC	118	79	8	0	445	17	93
EDP + 18MC	54	16	0	0	—	6	45

* The oldest EDP-treated test animals (alone or in combination) are 500 days of age. The oldest MC-treated mice are 700 days of age.

represent a threshold dose for this agent. Two groups of mice, one in the Bagg albino and the other in the CBA strain, each received a single dose of 200 r of x-rays at ages ranging from one to 70 days. Two groups from the same stocks received 14 weekly injections of 5 μg of estradiol dipropionate in peanut oil beginning at 42 days of age. In a third treatment series 2 groups received 200 r at 42 days followed by the 14 weekly injections.

Although 32% of untreated Bagg albino mice developed lymphomatous disease, the average age of appearance was 725 days, with the earliest spontaneous case being observed at 490 days. Only one of the 32 cases was a mediastinal (thymic) lymphosarcoma (Table I). In the CBA strain also (Table II) the average age of appearance of leukemia was late, 608 days, with only 2 of 20 spontaneous cases appearing before 515 days. None of these spontaneous cases was a mediastinal lymphosarcoma. Thus, out of a total of 52 spontaneous cases of lymphomatosis in these 2 stocks of mice only one was a mediastinal lymphosarcoma. The low incidence of mediastinal lymphosarcoma can be correlated with a delayed appearance of systemic lymphomatosis (leukemia). Where spontaneous leukemia appears relatively early, as in the F strain, the incidence of mediastinal lymphosarcoma is high.(7) Microscopic study of early mediastinal lymphosarcoma indicates that in the mouse the site of origin is within the thymus itself.(8) Thymectomy has decreased the spontaneous incidence.(9)

When threshold leukemogenic doses of x-

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TABLE III.
Occurrence of Lymphomatosis in dba Mice Receiving Threshold Doses of Methylcholanthrene and X-rays Either Singly or in Combination.

Agent	Total mice	Mice over 250 days	Total lymphoma	Mediastinal lymphoma	Age lymphoma (in days)	Dead and negative	No. living*
200r	78	38	0	0	—	13	69
18MC	105	94	21	2	325	47	37
200r + 18MC	97	78	29	14	320	20	48
Controls	117	117	19	3	350	95	0

* The oldest living mice are 700 days of age.

rays and estradiol dipropionate were given simultaneously to Bagg albino or CBA mice, synergistic effects were observed. Although a large per cent of the mice are still living (Tables I and II), of the 39 mice on combined treatment which have died 25 had lymphomatosis, and in 22 cases the disease was mediastinal lymphosarcoma.

Methylcholanthrene is not leukemogenic for these stocks.(6) When 18 skin paintings of 0.25% solution in benzene were combined with either 200 r of x-rays or 5 micrograms of estradiol dipropionate weekly for 14 weeks, the incidence of lymphomatosis was no greater than when either of the latter agents was administered alone (Tables I and II). In the dba stock, in which methylcholanthrene is leukemogenic,(3,6) the incidence of lymphomatosis was increased (Table III) when x-rays and the carcinogen were given simultaneously. The increased incidence can be accounted for solely by the greater number of mediastinal lymphosarcomas, the incidence of generalized lymphomatosis being the same whether methylcholanthrene was given alone or combined with x-radiation.

These experiments indicate that the thymus of young mice of certain strains contains lymphoid tissue which is very sensitive to the lymphomogenic action of certain agents. Although systemic lymphomatosis (leukemia) developed at an advanced age in the Bagg albino and CBA stocks, spontaneous thymic lymphosarcoma appeared only once in a population of 164 controls. Thus lymphocytic neoplastic transformation within the thymus represents a disease which appeared spontaneously only occasionally although it could be readily induced.

A total of 70 μg of estradiol dipropionate acted as a synergist in the induction of mediastinal lymphosarcoma. This amount of estrogen, when given from 42 to 140 days of age, was not sufficient to inhibit the ability of test animals to resume reproductive activity. In the development of spontaneous leukemia multiple factors may be operating synergistically so that significant causative ones may be obscured.

Summary. Synergistic lymphomogenic effects were obtained when estrogenic hormone and x-rays were administered simultaneously to mice of the Bagg albino and CBA stocks. Thymic lymphoid tissue appeared to be more sensitive than other lymphoid tissue. This was also true when x-rays and methylcholanthrene acted synergistically in the dba stock.

Received November 1, 1949. P.S.E.B.M., 1949, v72.

Liver Glycogen Response to Adrenal Cortical Extract of Diabetic and Non-Diabetic Rats.* (17523)

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This paper is a report of the liver glycogen response of alloxan-diabetic rats, following adrenalectomy, to adrenal cortical extract. It was of interest to determine to what extent liver glycogen could be increased by adrenal cortical hormones in the absence of an adequate insulin supply.

It has been previously reported(1-3) and confirmed in this paper (Table I), that the livers of alloxan-diabetic rats contain more glycogen than normal rat livers following a 24-hour fast. When fed(4-6) or comatose diabetic (2,7) animals have been used, a higher level of glycogen in the diabetic animal was not found. This difference in the liver glycogen levels of the fasted normal and diabetic animals did not complicate the experiment, however, since all animals were adrenalectomized 3 days prior to injection of adrenal cortical extract (ACE). The liver glycogen of the adrenalectomized diabetic rat (0.012 ± 0.002 mg/100 g liver) is of the same low order of magnitude as the adrenalectomized normal rat (0.016 ± 0.003 mg/100 g liver, Table II).

Experimental. Sprague-Dawley rats, male and female, weighing approximately 100 g (range, 80-110 g) were given alloxan (generally 130 mg/kg) subcutaneously following a 24 hour fast. For the following 24 hours, 5% glucose was substituted for drinking water. Nonfasting blood sugars were determined the following week by a modification of Folin's micromethod.(8) The animals were adrenalectomized 6-10 days later, and 3 days after adrenalectomy liver glycogen was determined by the method used by Dorfman *et al.*(9) Upjohn's lipo-adrenal extract (1 cc $\Bumpeq$ 2 mg 11-dehydro-17-OH-corticosterone) was used. In the first experiment (Table II) this was diluted 1:1 with mazola oil. Following a 24 hour fast, each of the injected animals received 4 subcutaneous doses of ¼ cc at 2 hour intervals, and all animals were sacrificed 2 hours after the last injection. The animals were divided into 4 groups: (1) adrenalectomized, (2) adrenalectomized-diabetic, (3) adrenalectomized plus ACE, (4) adrenalectomized-diabetic plus ACE; the results are tabulated in Table II.

The results indicate that there is no statistically significant difference between the liver glycogen response to ACE of diabetic and non-diabetic rats following adrenalectomy. If the diabetic animal has reduced liver hexokinase activity or other defect causing reduced phosphorylation of sugar, it is possible that glycogen is formed largely from carbon fragments smaller than hexose; this is in agreement with the interpretation by Stetten(10) of the defects in alloxan diabetes.

Summary. Liver glycogen of alloxan-diabetic rats was 8 times as great as in normal rats. Both groups were fasted 24 hours. The liver

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* Supported in part by a grant (RG 1050) from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service, and in part by a grant from Ciba Pharmaceutical Products, Inc.

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TABLE I.
Liver Glycogen in Normal and Diabetic Rats (Fasted 24 Hr).

Status	No. of animals	Liver glycogen, mg/100 g liver	P	Blood sugar, mg% (mean and range)
Diabetic	11	.65 ± .14	<.01	658 (403-1100)
Normal	13	.08 ± .03	>.4	
Non-diabetic (received alloxan)	6	.14 ± .07		129 (120-143)

TABLE II.
Liver Glycogen Following Administration of Adrenal Cortical Extract to Diabetic and Non-diabetic Rats. (All animals adrenalectomized and fasted 32 hr).

Status	No. of animals	Treatment	Liver glycogen, mg/100 g liver	P	Blood sugar,† mg% (mean and range)
Diabetic	14	0	.012 ± .002	>.2	410 (264-620)
Non-diabetic	17	0	.016 ± .003		
Diabetic	7	ACE*	.44 ± .08	>.9	380 (280-645)
Non-diabetic	9	ACE*	.43 ± .10		
Diabetic	9	ACE	1.67 ± .16	>.2	397 (296-556)
Non-diabetic	10	ACE	1.97 ± .16		

* Diluted 1:1. † Prior to adrenalectomy.

glycogen response to adrenal cortical extract of diabetic rats was found not to be significantly different from the response of non-diabetic rats. Both groups were adrenalectomized and fasted 32 hours.

Received November 3, 1949. P.S.E.B.M., 1949, v72.

Hemagglutination and Complement Fixation with Type I and II Albany Strains of Coxsackie Virus.[*] (17524)

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Dalldorf and Sickles(1) isolated a virus from the feces of patients having symptoms resembling those of abortive or even paralytic poliomyelitis. This active agent, characterized by pathogenicity for suckling but not for adult mice, was later named Coxsackie virus.(2) Melnick, Shaw, and Curnen(3) recovered several similar viruses some of which they found to be distinct by the neutralization test. Dalldorf(4) also determined such specificity for his strains, here designated as Type I and Type II Albany strains. The difficulties of working with infant mice are considerable; we were thus led to a study of a possible *in vitro* reaction that might be applicable for the identification of the virus, namely, hemagglutination or complement fixation. The present report concerns itself with the results of studies of the two reactions employing 2 of the Albany strains, Type I (T.T.) and Type II (Fleetwood), generously presented to us by Dr. Dalldorf.

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[*] With the technical assistance of Joan FitzGerald and Barbara Quinn.

[†] Major, V.C., U. S. Army.

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Hemagglutination Tests. Agglutination tests were carried out in a manner already described,(5) except that the saline solution used for dilution of materials was buffered with 0.02M, instead of 0.05M phosphate. Human group O, sheep, chick, and guinea pig RBC were employed and the two different strains, Type I and II, of the Coxsackie virus. In addition, two forms of each strain were studied; suspensions of infected brain and of limbs, derived from infant mice which had reacted after their injection intraperitoneally of either active brain or limb virus.(6) The tubes containing materials were held at 5, 23, and 37°C and read at intervals up to 2 hours. As an indicator of the possibility of the materials yielding positive reactions, a test was set up with sheep cells and MM virus; definite agglutination was seen in 1:160 dilution of the virus after 1 hour at 5°C.(5) The results of all the hemagglutination tests with the 2 strains of Coxsackie virus were, however, negative. It should be stressed that suspensions of normal mouse brain, especially of fresh brain, if not properly centrifuged, show agglutination of sheep RBC, the degree of hemagglutination depending on the particles in the supernate.

Complement-fixation tests. Complement-fixation tests have been carried out with the same Albany strains using mouse immune serum and antigens derived from infected brain or limb tissue of suckling mice, Swiss-W strain.‡

Immune Serum. Antisera were prepared in 3- to 4-month-old Swiss-W mice by giving each of them, intraperitoneally, 0.5 ml of mouse-brain virus, 10^{-1} dilution, on the 1st and 5th day; they were bled on the 15th day. These animals were then injected intraperitoneally with limb virus, each with 0.5 ml of 10^{-1} dilution, on the 19th and 25th day; they were bled again on the 35th day. In all 20 mice were so treated with each type of virus.

Antigen for test. Antigens for complement-fixation tests were prepared following a method previously described (acetone-ether extracted antigens).(7) In the present study, 2- to 11-day-old Swiss mice were given .05 ml of 10^{-1} suspension of infected brain or limb tissue intraperitoneally. When definitely ill,‡ usually on the 3rd day, brain and limb tissue, the skin being removed from the latter, was collected separately and prepared(7) as antigens. It should be stated here that the titer of virus in limb tissue derived from 11-day-old mice was greater than $10^{-7.5}$, confirming the observations of Gifford and Dalldorf.(2) Extraction of the limb tissue with acetone-ether was found to be more satisfactory than the method of suspension in saline solution followed by centrifugation at 12,000 rpm. For control material, brain and limb tissue was collected from apparently normal Swiss mice of the same age. The complement-fixation test was performed in the manner previously described.(8)

Experimental. Table I illustrates one of the tests. A specific fixation was obtained with both types of Coxsackie virus, used as limb-tissue antigen, without any demonstrable sign of cross reaction. Moreover, antigens made up of brain-tissue virus were not effective. Indeed, following the injection of brain virus into mice, their sera were either negative or of a low titer; whereas after immunization with limb-tissue virus a high titer of fixing antibody was obtained. It would appear that the limb-tissue virus is a highly potent antigen. Finally, no reaction took place between Cox-

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TABLE I.
Showing Complement-fixation Tests with Albany Strains of Coxsackie Virus.

Antigen	Serum Type I brain	Type I limb	Type II brain	Type II limb	Normal	Western equine
Type I limb	0	1/16	0	0	0	0
Type I brain	0	0	0	0	0	0
Type II limb	0	0	1/2	1/32	0	0
Normal limb	0	0	0	0	0	0
Western equine brain	0	0	0	0	0	1/64

Fractions represent the highest dilution of serum showing 2+ or better complement fixation; in the test, serum was diluted 2-fold beginning with 1:2 dilution.

sackie-virus sera or antigens and unrelated virus or apparently normal materials.

Summary. Two Albany strains of Coxsackie virus, Type I (T.T.) and Type II (Fleetwood) were found to be negative for hemagglutination when human O, sheep, chick, and guinea pig erythrocytes were used. On the other hand, the viruses showed distinct positive specific complement fixation without any cross reaction between them. Antigens prepared from infected brain tissue were inactive; those from infected limb tissue were highly active. Tests with human sera and limb-tissue antigens are left for further study.

Received November 4, 1949. P.S.E.B.M., 1949, v72.

The Pituitary-Adrenal Relationship in the Infant Rat. (17525)

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The administration of adrenocorticotrophic hormone (ACTH) to the rat or guinea pig results in an almost immediate fall in adrenal ascorbic acid content and a more gradual decline in adrenal cholesterol.(1,2) This has been correlated with evidence of adrenal cortical secretion.(3) It had been previously demonstrated by Selye(4) that exposure to stress or the injection of noxious substances causes the secretion of ACTH by the adenohypophysis of the intact animal. Exposure of animals to these same conditions causes a depletion of adrenal ascorbic acid in the intact animal but not in the hypophysectomized animal.(5) The purpose of this investigation was to determine whether the newborn rat could respond to stress and the administration of ACTH in a manner similar to that of the adult.

Infant rats at 2, 4, 6, 8 and 10 days of age were injected with 0.04-0.05 mg of aqueous epinephrine and the adrenals removed one to two hours later. In all, 170 rats from 27 litters were used in this study. Adrenal ascorbic acid was determined by the method of Roe and Kuether.(6) It was found that no decrease in adrenal ascorbic acid occurred until

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TABLE I.
Effect of Administration of Epinephrine and Refrigeration on Adrenal Ascorbic Acid Content.

Age, days	ascorbic acid, % of control value Epinephrine	 Refrigeration
2	93	
4	98	95
6	91	101
7		105
8	69	
10	66	
11		105
14		107
16		83
25		81
Adult	62	

TABLE II.
Effect of 2.5 Mg ACTH on Adrenal Ascorbic Acid Content.

Age, days	ascorbic acid, % of control value
4	66
6	64

the animals had reached 8 days of age. (Table I). Exposure of animals to refrigeration (5°C) for 2½ hours similarly did not result in a decline in the ascorbic content until the 16th day of life. With this stress the fall in ascorbic acid was 19% or approximately one-half of that with epinephrine (Table II).

Since very young rats do not respond to stress, it was necessary to further localize the point of insensitivity, *e.g.*, the pituitary or adrenal. Therefore, rats of 4 and 6 days of age were injected intraperitoneally with 2.5 mg† ACTH and killed 80-120 minutes later. Ascorbic acid analysis of the adrenals revealed a 34-36% decline in the vitamin C content under these conditions (Table II).

It would appear from these results that the fall in adrenal ascorbic acid in the newborn rat does not occur as a result of exposure to stress at it does in the adult animal. Since the adrenals are capable of responding to the administration of ACTH at this age, it is probable that the adenohypophysis is the organ which is not capable of responding to stress by secreting ACTH until the animal has reached a certain state of maturation.

Summary. The administration of epinephrine to the infant rat does not result in a fall in adrenal ascorbic acid until the animal reaches the eighth day of life. Under the conditions of the experiment, adrenal ascorbic acid does not decline as a result of refrigeration until the 16th day of life. The administration of ACTH, on the other hand, causes adrenal ascorbic acid depletion on the 4th and 6th day of life.

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† Armour ACTH, kindly supplied by Dr. John R. Mote, Medical Director of Armour Laboratories.

Received November 7, 1949. P.S.E.B.M., 1949, v72.

Labeling of Rat Hemoglobin with Radioactive Iron.* (17526)

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In the studies of the biological aspects of iron metabolism, radioactive iron serves as a suitable means of following, *in vivo,* transformations of forms of iron. Especially valuable is its use in tagging hemoglobin molecules so that, under certain conditions, hemoglobin iron, hemoglobin molecules or red cells may be identified as they undergo certain chemical or physical changes. The current methods(1-7) used for incorporating

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radioactive iron as a heme constituent in hemoglobin often present major difficulties. Most iron compounds have a high toxicity and low stability which limits their use. The low specific activity and soft beta radiation of the available radioactive iron make it necessary to obtain a high percentage of tagging for most studies. Finally, since synthesis can be accomplished only in donor animals, preliminary production of anemic animals with drastically reduced iron stores is a time consuming and, at times, unpredictable procedure involving many serial bleedings and careful diet considerations.

In the course of iron studies in our laboratory, we have developed a procedure which we feel will provide an easier and, perhaps, a more satisfactory tagging of hemoglobin with radioactive iron than can be achieved with the methods which have been used. The iron used contained a mixture of Fe-55 and Fe-59 prepared by pile bombardment of enriched Fe-58. Concentrated hydrochloric acid, in amount approximately twice the molarity required for quantitative reaction with the iron sample was diluted with 3 parts of distilled water and poured over the powdered iron in a large flask. The reaction was allowed to continue, with heat supplied as the reaction slowed, until it was complete as indicated by a clear green solution without significant insoluble residue. This solution of ferrous chloride in excess hydrochloric acid was filtered, a sample analyzed by the Wong thiocyanate method(8) for iron and the remainder stored in a glass stoppered stock bottle. Working solutions were prepared by withdrawing from the stock the amount of iron desired and placing it in an appropriate volumetric flask. The HCl and water were removed by evacuation and heat. The green ferrous chloride powder was dissolved in 0.1 N lactic acid and the flask filled to the mark with this acid. The concentrations of ferrous chloride and lactic acid were considered in terms of volume which would be desirable for injection, the amount and concentration the animal could tolerate and the relative molarities of the iron and lactate which would provide a pH and reducing environment for the iron suitable for prolonged storage at room temperature. These conditions were met by preparing a 400 mg % Fe solution of ferrous chloride in 0.1 N lactic acid. The solution was sterilized by Seitz filtration and stored in a sterile rubber-capped bottle at room temperature. Such working solutions show no signs of deterioration after more than two months of storage.

Adult male and female white rats were used. Their weights ranged between 225 and 330 g. By cutting the tails and suspending them in graduated centrifuge tubes containing warm sodium citrate, the animals were bled about 1.5% of their body weight at 2-3 day intervals for 11 bleedings. Their hematocrit values dropped to an average of about 25%. At this point, iron injections were begun and during the course of the injections four more bleedings were performed. Also the usual Rockland Rat diet was supplemented by milk at this point. Response by weight gain, increased appetite and general vigor was apparent within one week. At no time were they maintained on a diet considered low in iron.

The working lactic acid-iron solution was injected into rats intraperitoneally in doses of 0.25 cc per 100 g of body weight, which provided 1 mg of iron per 100 g of body weight. These injections were given daily for the first half and every other day for the remainder of the series. A total of 14 injections were given, of which the third and tenth injections were at one-half dose. The total amount of iron thus administered was 13 mg/100 g of body weight. This is notably in excess of the animal's iron requirement.

The low toxicity of the injections is evident in animals in good health. Of 12 rats, 4 deaths occurred after iron injections were begun. One of the deaths occurred as a result of the 12th bleeding. The other 3 followed the 2nd, 7th and 9th injection of lactic acid-iron solution. Although peritonitis could not be excluded because of the irritation inflammation produced by the acid, death followed not

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TABLE I.
Percentage Tag of Hemoglobin with Radioactive Iron. The stock radioactive iron injected gave 3,300 counts/min./mg of iron. The values represent no duplicates since the blood was collected by exsanguination and pooled for each lot.

Sample	No. animals	Days since last inj.	Counts/min./mg Fe	% tag
4	3	—2	957	29.0
5	2	1	850	25.8
6	2	3	978	29.7
7	1	19	988	29.8

earlier than 12-24 hours after the injection.

Radioactivity determinations were made by counting desiccated protein-free filtrates and chemical iron was determined on such filtrates by the method of Wong.(8) Counts were sufficiently high under the geometric and sensitivity conditions employed to allow accurate comparison of samples.

In Table I are recorded the percentages of tagging achieved by our method. To be noted is the reasonable consistency of the values, each of which were obtained from a separate group of animals. Once bleedings were stopped, then no appreciable increase in tagging seemed evident over a 3-week period. After iron injections were begun, but before bleedings were stopped, a definite increase in tagging was apparent at weekly intervals.

Although we have demonstrated that a 25-30% tag may be accomplished in 48 days, in rats, we feel that the institution of milk supplement to the diet earlier in the experiment would have eliminated some of the emaciation. Furthermore, the distress observed in the animals with the first few injections might have been eliminated. We also feel that satisfactory tagging could be accomplished with smaller doses, thus minimizing adhesions and iron staining of the viscera which was noted at autopsy. It is believed that if a greater percentage of tagging was desired it could be accomplished by continuing the serial bleeding and iron injections for a longer period of time.

Conclusions and summary. The tagging of hemoglobin in the red cells of rats by the method described is efficient. The iron compound, ferrous chloride, is of low toxicity, the lactic acid in which it was dissolved provides a satisfactory stabilizing environment and the solution is well tolerated when injected. The data show a consistency in the percentage of tagging as evidence of the reproducibility of the experiment. In addition to the ease with which the materials are prepared, the short duration of the experiment is a desirable feature.

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Received November 7, 1949. P.S.E.B.M., 1949, v72.

Effects of Hemorrhagic Shock upon the Electroencephalogram. (17527)

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The central nervous system is extremely sensitive to reduction of its oxygen supply, particularly the small pyramidal cells of the cerebral cortex.(1) In view of the general acceptance of this fact, it is surprising that studies have not been made of the effects of anemic anoxia produced by hemorrhage upon the electrical activity of the cerebral cortex as determined by the electroencephalograph.

Materials and methods. Rabbits weighing 5 to 11 lb were used in these experiments. General anesthesia was not used. Curare (In-

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tocostrin) was employed to abolish skeletal muscular movements which affect the electroencephalogram. Curare was used in a dose of 0.7 unit per lb. of body weight. The 4 electrodes of the electroencephalograph were placed in similar positions on the heads of the animals throughout these studies. The right femoral artery was exposed under local anesthesia (5 cc of 1% novocaine solution), cannulated and attached to a blood pressure recording manometer. Shock was produced by graded hemorrhage. The blood which was withdrawn was prevented from clotting by the addition of heparin solution. The heparinized blood was returned to the animals in many of the experiments.

Results. In control runs before curare was given, the electroencephalograms revealed a basic frequency of 6 to 7 cycles per second with an amplitude averaging about 75 microvolts. Numerous variations in the amplitude and frequency of the waves occurred through the record. There were short flurries of low voltage and of 20 to 30 cycle activity, and occasional sharp waves and spikes of 100 microvolts were observed. Following injection of curare, the rapid activity disappeared leaving a fairly symmetrical rolling type of wave, varying in frequency between 4 to 7 cycles per second and having an average amplitude of 50 microvolts.

Moderate to deep shock produced by bleeding resulted in slowing of the wave frequency to an average of 2 to 4 cycles per second. At the same time the amplitude of the waves increased to 150 to 200 microvolts and in some animals reached 400 microvolts. This was the basic pattern. There were, however, many irregular waves, sharp waves, rolling waves and occasional dicrotic or notched waves. During recovery from shock the slow waves with high amplitude were replaced intermittently by rapid waves (10 cycles) of lower amplitude (100 microvolts).

If shock were progressive and transfusions were not given, the slow rolling waves persisted but their amplitude decreased. The voltage diminished and finally the wave pattern disappeared corresponding with the death of the animal. In those animals which survived without transfusion (reversible shock) the basic pattern of slow rolling waves of increased amplitude was maintained until the conclusion of the experiment. In shock reversible by blood transfusion the wave pattern returned (sometimes within 10 minutes) to the type present before bleeding. The blood pressure returned to normal before the electroencephalogram. In shock irreversible by blood transfusion, the waves decreased in amplitude and frequency despite the administration of blood. It was noted in these experiments that the response of the electroencephalogram was a better prognostic criterion than was the response of the blood pressure. A persistent decrease in the frequency and amplitude of the waves was an ominous sign even though the blood pressure rose following blood transfusion. The longer the duration of shock, the more difficult was it to restore the electroencephalogram to normal with blood transfusions.

Discussion. The mechanism of production of the electroencephalographic changes in hemorrhagic shock may be briefly considered. Slow waves of increased amplitude have been observed in hypotension due to carotid sinus syncope,(2) insulin shock,(3) in anoxemia,(4) and after ligation of the carotid arteries for an intracranial saccular aneurysm.(5) The effect of low blood pressure due to stimulation of the vagus in depressing cortical electrical activity has been observed to be almost identical with that due to anoxemia.(6) It is suggested, therefore, that the electroencephalographic changes in hemorrhagic shock are due, in part at least, to cerebral anoxia.

Summary and conclusions. Hemorrhagic shock in rabbits produces definite changes in the electroencephalogram. These are characterized basically by the appearance of slow

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waves of increased amplitude. In shock irreversible to transfusion there occurs a progressive decrease in cortical electrical activity which is shown on the electroencephalogram as a continued reduction in the frequency and voltage (amplitude) of the wave pattern. The effects on the electroencephalogram of blood transfusion in reversible and irreversible hemorrhagic shock are discussed. It is tentatively suggested that the electroencephalographic response to blood transfusions in shock may be used as a means of differentiating the reversible and irreversible forms.

Received November 8, 1949. P.S.E.B.M., 1949, v72.

Vitamin B_{12b}: Some Properties and its Therapeutic Use.* (17528)

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The preparation of two red fractions from liver extract,(1) both clinically active in pernicious anemia, was reported by Smith who suggested that one fraction might arise from the other by proteolysis. In later publications(2,3) it was reported that paper chromatography enabled 2 red factors to be recognized in liver extract. Both of these were active for *L. lactis* Dorner. In our investigations it was found that concentrated liver extract yielded a pink fraction by chromatography with silicic acid. Upon purification, this fraction yielded a red pigment with an absorption spectrum differing from that of vitamin B_{12}. An apparently similar fraction was obtained from cultures of *Streptomyces aureo-*

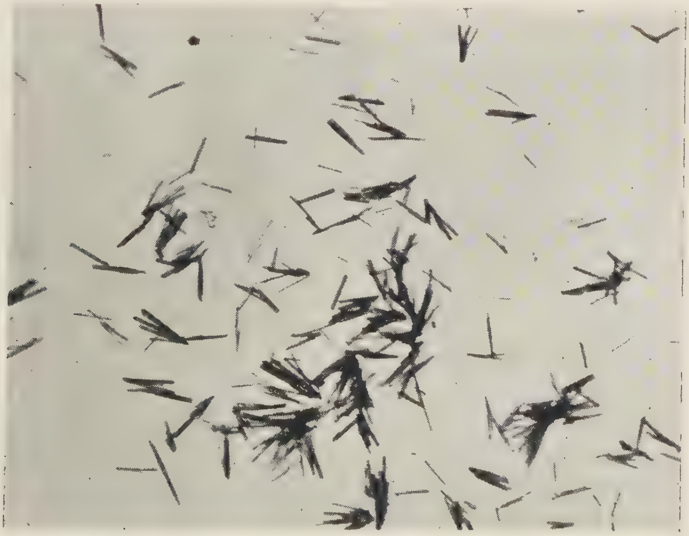

Fig. 1.
Crystals of vitamin B_{12b} ×425.

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* Paper presented at the Eastern Section of the American Federation for Clinical Research at Boston, Mass., on Dec. 3, 1940.

The expenses of this investigation were defrayed by a Lederle grant.

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TABLE I.

Vitamin B_{12b}: Hematological Response in Patients with Addisonian Pernicious Anemia.

Case No.	1	2	3	4	5
Vit. B_{12b}, γ, I.M. daily	1	1	1	1.5	2
Initial RBC, mil/cmm	1.90	2.60	1.74	1.75	1.47
Day of reticulocyte peak	8	8	9	7	9
Max. reticulocytes, %	11.4	14.0	18.0	8.3	15.1
Expected max. retic., %	18	5	18	18	24
RBC, mil/cmm, at 3 wk	3.06*	4.36	2.84	3.17	2.95
Expected RBC, at 3 wk	3.11*	3.43	3.19	3.29	3.17

* At 2½ wk.

faciens and red crystals of a biologically active compound "vitamin B_{12b}"(4) were obtained from this fraction. The crystals of vitamin B_{12b} are shown in Fig. 1. In the present investigation crystalline vitamin B_{12b} was prepared from cultures of *S. aureofaciens* by absorption on charcoal, precipitation with ammonium sulfate, chromatography upon silicic acid columns and crystallization from acetone-water mixtures. The vitamin was dissolved in 0.9% sodium chloride solution at pH 7.0. The solution was sterilized by filtration through a Seitz filter. The final concentration of vitamin B_{12b} was adjusted to 10 μg per ml.

Clinical Observations. The solution of crystalline B_{12b} containing 10 μg per ml was used for assay in 5 patients with Addisonian pernicious anemia. All patients had megaloblastic bone marrows and histamine-fast achlorhydria. The age range was 55 to 75 years. Vitamin B_{12b} was administered by intramuscular injection daily for 21 days in the dosage schedule given in Table I. Hematologic response seemed to be optimal whether the amount given was 1, 1.5, or 2 micrograms. One patient had a higher reticulocyte peak than expected, one the same and three somewhat lower. The relative unreliability of the reticulocyte count as a quantitative index of the formation of red blood cells has been recently emphasized by several observers.(5,6) In contrast to the fluctuation of the reticulocyte peak in relation to that of the expected standard, the erythrocyte regeneration was within maximal limits in all 5 patients at the end of 21 days of therapy. It thus seems that 1 to 2 μg of vitamin B_{12b} is hemopoietically equivalent to 1 U.S.P. unit of liver extract and is then comparable to the potency of vitamin B_{12}.(6,7)

The detailed hematologic data of Case 2 who showed the most dramatic response are presented in Fig. 1. Therapy resulted in the usual reversal of the megaloblastic marrows to normal normoblastic cellularity. The white blood cell counts rose from leukopenic to normal figures with a loss of hypersegmentation of the neutrophils. In the only patient (Case 3) who has been maintained longer than 3 weeks on vitamin B_{12b}, the red cell count has risen to a nearly normal level at the end of 7 weeks.

Clinical improvement was also very satisfactory, beginning on the 3rd, 4th or 5th day of treatment, and was characterized by improved appetite and a sense of well-being. Severe glossitis in one patient completely subsided within 10 days. Neurological symptoms such as paresthesias disappeared in all cases. A patient who had advanced combined system disease had striking improvement within 3 weeks, including the reversal of a strongly positive Romberg's sign.

Oral administration of 5 μg of vitamin B_{12b} daily for 10 days was undertaken with a 6th patient with Addisonian pernicious anemia, having an initial red cell count of 2,090,000 per cmm. There was no response, the reticulocytes varying from 0.4% to 1.4%. The patient was then given the same therapy with

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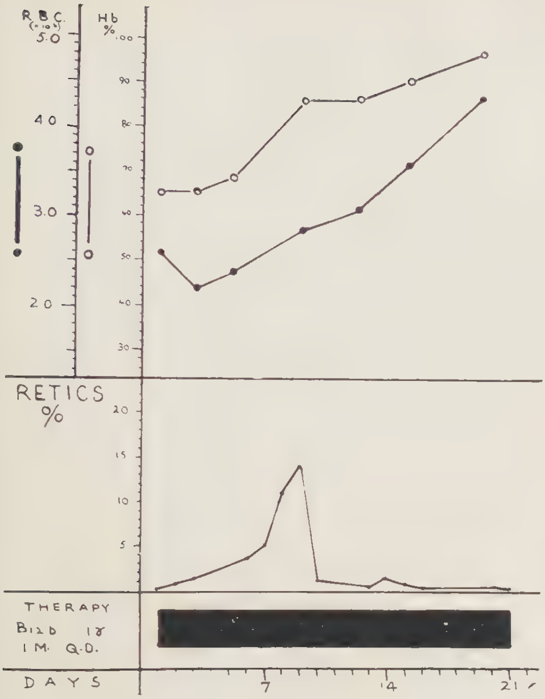

Fig. 2.
Hematological response of a patient with Addisonian pernicious anemia to vitamin B_{12b}. (Case 2).

the simultaneous addition of 150 ml of neutralized gastric juice daily for 12 days. The reticulocytes began to rise slowly on the 8th day to reach a peak of 3.4% on the 11th day. There was a concomitant red blood cell rise of 240,000 per cmm during this time, and slight but definite clinical improvement occurred. This is similar to the results obtained by Berk *et al.*(8) using vitamin B_{12}.

We observed one patient with megaloblastic anemia of pregnancy who had failed to respond to 3 weeks of therapy with 150 U.S.P. units of refined liver extract intramuscularly, and also showed no hematologic response to 1 μg daily of vitamin B_{12b} given intramuscularly in a 10 day period. The severe glossitis, unaffected by liver therapy, cleared completely on B_{12b}, and later there was a maximal hematologic response to pteroylglutamic acid. Several similar vitamin B_{12} refractory cases have been reported. The problem of different types of deficiencies occurring in megaloblastic anemias other than Addisonian pernicious anemia has been recently reviewed by Mueller *et al.*(9)

Summary. A pink fraction obtained from liver and from *Streptomyces aureofaciens* has been separated from vitamin B_{12} by silicic acid chromatography and has been shown to have different ultraviolet and visible absorption spectra. This new substance, vitamin B_{12b}, has been prepared in crystalline form and has been found to be effective parenterally in the treatment of patients with Addisonian pernicious anemia in amounts of 1 to 2 μg daily.

We are indebted to Mrs. Helen Jakubowski for performing the blood examination.

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Received November 9, 1949. P.S.E.B.M., 1949, v72.

Sterols and Fatty Acids in the Nutrition of Entozoic Amoebae in Cultures.* (17529)

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The many excellent media which have been suggested for culture of entozoic amoebae suffer from one serious deficiency when used for quantitative studies. Statistically satisfactory reproduction of results is the exception rather

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* Part of a project supported by a grant from the Division of Research Grants and Fellowships of the U. S. Public Health Service.

TABLE I.
Relative Growth of *E. histolytica* with Various Serum Fractions.

		Serum fractions. % of total								
		A			B			C		
Transfer	Control (LSB)	10	5	1	10	5	1	10	5	1
1	4+	3+	2+	+	4+	3+	3+	3+	2+	2+
2	4+	3+	3+	2+	4+	4+	2+	+	+	+
3	4+	2+	2+	+—	4+	4+	3+	+	+	+
4	4+	+	+	+	3+	4+	3+	+—	+—	+—
5	4+	+—	+—	—	3+	3+	4+	—	—	—
6	4+	—	—	—	3+	3+	3+	—	—	—
7	4+	—	—	—	4+	4+	4+	—	—	—
8	4+	—	—	—	4+	4+	4+	—	—	—
9	4+	0	0	0	3+	4+	4+	0	0	0
10	4+	0	0	0	4+	4+	3+	0	0	0
11	4+	0	0	0	4+	3+	3+	0	0	0

4+ = 40+/1pf.
3+ = 30-40/1pf.
2+ = 15-30/1pf.
1+ = 1-15/1pf.
+— = less than 1/1pf.
— = No detectable growth.
0 = Series discontinued.

than the rule. A fluid medium of constant composition is an absolute requirement. For several years we have used a modification of the Cleveland and Collier(1) and Chang(2) media in conjunction with physically purified rice starch. The medium (LSB) and method of preparing the starch have been described in a previous paper.(3) A necessary component of the LSB medium was serum. Since this was necessarily obtained in the form of whole blood from an abbatoir we were faced with the tedious procedure of separation, clarification and sterilization by filtration. The final medium was also sterilized by filtration with the attendant risk of loss by contamination and of alteration of the bacterial flora of amoeba cultures when a lightly contaminated tube of medium was inadvertently used.

As Reardon and Rees(4) had shown that serum was not required in the over-lay for whole egg media and that cholesterol could substitute in part for egg yolk(5) we attempted without success to replace the serum of LSB medium with the recommended amount of cholesterol. We therefore subjected serum to fractionation.

Experimental. The serum proteins were precipitated by adding 5 vol. of ethyl alcohol at 0°C. The precipitate was exhaustively washed with cold alcohol and cold acetone. The dried precipitate was redissolved in M/30 phosphate buffer in 0.4% NaCl at pH 7.2 and brought to the volume of the original serum, (Fraction "A".) The pooled filtrates were evaporated to dryness and the residue extracted with absolute ethyl alcohol. The extract was reduced to 10 ml volume and added rapidly to a volume of buffered NaCl solution equal to that of the original serum. The alcohol was removed by boiling and the original volume restored with distilled water. The resulting emulsion was Fraction "B." The alcohol-insoluble residue was extracted with acetone and an emulsion prepared as above (Fraction "C".) The 3 fractions so obtained were added to the liver-infusion base of LSB medium in proportions corresponding to 10, 5 and 1% of whole serum. The medium containing Fraction "A" was sterilized by

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filtration, the others in the autoclave for 15 minutes at 15 lbs.

All media were inoculated with the NRS strain of *Endamoeba histolytica* using the usual technic. The cultures were incubated at 37°C, examined and transferred at 48 hour intervals with the results shown in Table I. With the extracted proteins growth failed beyond the fourth or fifth transfer on repeated trials. The alcohol-insoluble, acetone-soluble fraction was even less effective. The alcohol soluble fraction, however, was apparently capable of supporting growth indefinitely. These results suggested that steroid or phospho-lipid components of serum might be the needed factor in spite of our previous failure to get persistent growth when serum was replaced with pure cholesterol. The basic liver infusion and completed medium (LSB) were analyzed for cholesterol and cholesterol esters by the method of Hess.(6) The infusion contained 0.023 mg cholesterol per ml and no detectable esters. LSB medium had 0.071 mg cholesterol and 0.130 mg cholesterol esters per ml.

We therefore substituted 0.15 mg per ml of cholesteryl oleate or palmitate for the serum of LSB medium, sterilized the media in the autoclave and tested them with the NRS strain as above. The cholesteryl oleate medium gave results at least equal to the serum medium and was perfectly clear though slightly opalescent.

As we refined our quantitative technic it became possible to assay the individual effects of components of the medium. We therefore tested the segregated action of the cholesterol and oleic acid moieties singly and in combination. Media were prepared containing cholesteryl oleate 0.15 mg per ml, cholesterol 0.09 mg per ml, oleic acid 0.06 mg per ml and the mixture of the last two. These were inoculated in duplicate with 100,000 trophic amoebae from a 48 hour culture of a strain of *Entamoeba terrapinae,* incubated for 48 hours at 30°C, and the yields determined. Subcultures were similarly inoculated where the yield was sufficient to provide 100,000 organisms, otherwise the entire sediment was transferred. *E. terrapinae* has been used for most of our quantitative studies as its growth parallels that of *E. histolytica* without being as sensitive to changes in the environment.

TABLE II.
Comparison of Cholesteryl-Oleate and Cholesterol Media.
Mean yields of duplicate cultures × 10^{-4}.

	Media		
Transfer	Cholesteryl-oleate	Cholesterol	Mean
1	165	216	190
2	184	204	194
3	186	229	207
4	150	194	172
5	179	188	184
6	179	212	196
7	173	165	169
8	180	258	217
9	157	226	192
Mean	172	210	191

In the media containing oleic acid alone or mixed with cholesterol there was no growth. The initial inoculum was not recoverable and the cultures were free of amoebae after the second transfer. The results obtained with the successful media are given in Table II. Direct comparison with LSB medium was not possible since it had been abandoned in favor of the cholesteryl oleate medium. However, 40 similar cultures in LSB medium selected at random from previous work gave a mean yield of 173 x 10^4. The analysis of variance gives little reason to question the greater effectiveness of the cholesterinized medium (F = 51.76) and indicates that our previous failure to obtain growth with cholesterol alone was due to inadequate concentration. Transfers and interaction introduced some variability (F = 3.66 and 3.18 respectively) which can be traced to instability in the cholesterinized medium. The yields in cholesteryl oleate medium are notably homogeneous in successive transfers (F = 0.86) in contrast to those in cholesterinized medium (F = 9.09).

The unexpected finding that oleic acid by itself and in mixture with its equivalent of cholesterol was extremely toxic led us to a study of the quantitative nature of its action. Cholesterinized liver infusion was mixed with

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TABLE III.
Effect of Oleic Acid on Growth of *E. terrapinae*. Mean yields of duplicate cultures $\times 10^{-4}$.

	Oleic acid, mg/ml						
Transfer	0.00	0.01	0.02	0.03	0.04	0.05	Mean
1	510	694	765	610	498	293	560
2	438	377	819	430	202	361	438
3	460	387	670	240	169	174	350
4	514	307	515	452	274	235	383
5	404	371	593	370	288	178	368
Mean	464	427	672	420	286	248	420

graded amounts of oleic acid up to 0.05 mg per ml and the media inoculated with 100,000 amoebae as before. The results over successive transfers are given in Table III. The highly significant effects are associated with concentrations of oleic acid (F = 33.00) and the comparison between the initial transfer and those following (F = 45.01). The slight heterogeneity existing among the second through fifth transfers and their interaction with concentration (F = 2.64 and 2.28 respectively) reflects the progressively poorer yields with oleic acid concentrations other than optimum.

Discussion. Our initial failure to substitute cholesterol for serum in LSB medium appears to be due to a quantitative effect. When supplied with less than some undetermined minimum amoebic growth fails. The stimulating effect of oleic acid in optimum concentration (0.02 mg per ml), together with the smaller yields obtained with cholesteryl oleate, suggests that cholesteryl oleate as such is not available to the amoebae but must be hydrolyzed to its constituent molecules.

Summary. Cholesterol in adequate amounts can be substituted for serum in liver infusion media for culture of entozoic amoebae, but the yields will be statistically heterogeneous in contrast with those obtained when cholesteryl oleate is used as a supplement. Oleic acid in optimum concentrations reinforces the action of cholesterol. It is suggested that cholesteryl oleate is not used as such but must be hydrolyzed to its constituent molecules.

Received Sept. 27, 1949. P.S.E.B.M., 1949, v72

Beneficial Effects of Liver on Growth and Survival of Immature Rats Fed Lactose-Containing Diets. (17530)

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Available data indicate that the deleterious effects of rations containing high levels of lactose are dependent in considerable degree on the composition of the diet employed. Boutwell *et al.*(1) observed that rats fed a purified ration containing 48% lactose and 28% corn oil developed an unthrifty appearance and extensive diarrhea during the first 6 to 12 days of feeding. A similar ration containing 28% butter fat in place of the corn oil resulted in better growth, larger food intake and a good outward appearance. Furthermore, animals fed the butter fat ration had considerably less diarrhea than those on the corn oil diet.(2) A similar difference between animals fed butter fat and corn oil was observed by Ershoff and Deuel(3) in rats fed diets consisting solely of 70% lactose and 30% fat. Animals fed butter fat or margarine fat in conjunction with 70% lactose lived significantly longer than those fed a similar ration containing corn oil or cottonseed oil as the source of dietary fat. On a natural food ration, however, in which lactose was provided in the form of skim milk powder, no significant difference in growth was observed between rats fed butter

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TABLE I.
Composition of Experimental Diets.

Dietary component	Diet A_1	B_1	A_2	B_2	A_3	B_3	A_4	B_4
Lactose*			30.2	30.2	45.3	45.3	60.5	60.5
Sucrose	70.5	60.5	40.3	30.3	25.2	15.2	10.0	
Casein†	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0
Salt mixture‡	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Whole liver powder§		10.0		10.0		10.0		10.0

To each kg of the above diets were added the following synthetic vitamins: Thiamine hydrochloride, 72 mg; riboflavin, 9 mg; pyridoxine hydrochloride, 15 mg; calcium pantothenate, 67.2 mg; nicotinic acid, 60 mg; 2 methyl-naphthoquinone, 5 mg; and choline chloride, 1.2 g. Each rat also received 3 times weekly the following supplement: Cottonseed oil (Wesson), 500 mg; α-tocopherol acetate, 1.5 mg; and a vitamin A-D concentrate‖ containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.

* Milk Sugar Merck, Merck and Co., Inc., Rahway, N. J.

† Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

‡ Sure's Salt Mixture No. 1.[9]

§ Whole Dried Liver Powder, Armour and Co., Chicago, Ill.

‖ Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per g.

or vegetable fats.(4-6) Findings by Boutwell *et al.*(2) indicate that the superiority of butter fat over vegetable fat on a lactose-containing diet is dependent on the B vitamin content of the diet. Animals fed butter fat gained significantly more weight than those fed vegetable fats when the basal diet was low in B vitamins. When the B vitamin content of the diet was increased, however, no significant differences in growth were observed on any of the diets employed. Boutwell *et al.*(2) suggest that butter fat may be superior to vegetable fats in promoting the intestinal synthesis of B vitamins or other nutrients essential for optimal lactose utilization. In line with the above Cary and Hartman(7) observed that liver contains a water-soluble factor (distinct from any of the known B vitamins with the possible exception of vitamin B_{12}) which significantly increased the growth of immature rats fed a purified ration containing 27% lactose. Liver has similarly been found to exert a protective effect on the growth and length of survival of immature rats fed a diet containing 73.2% β-lactose.(8) The present experiment was undertaken to obtain further data on the effects of liver feeding on growth and length of survival of immature rats fed purified rations of varying lactose content.

Procedure and results. Two basal rations were employed in the present experiment: diet A and diet B. Diet A was a purified ration containing the B vitamin factors in synthetic form only; diet B was similar in composition but contained in addition 10% defatted and desiccated whole liver. Both rations were supplemented with 0.0, 30.2, 45.3 and 60.5% U.S.P. lactose added in place of an equal amount of sucrose. Sixty-four female rats of the Long-Evans strain were selected at 22 to 24 days of age and an average weight of 44.7 g for the present experiment. Animals were placed in metal cages with raised screen bottoms to prevent access to feces and were fed *ad lib* the various diets listed in Table I (8 animals per group). Feeding was continued for 40 days or until death, whichever occurred sooner. All surviving rats were autopsied after 40 days of feeding. Ovarian weights were determined; ovaries were fixed in 10% formol, and sections were prepared and stained with hematoxylin and eosin.

Findings indicate that growth was retarded in all rats fed lactose-containing rations

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TABLE II.
Effects of Liver Feeding on Body and Ovarian Weight and the Length of Survival of Immature Rats Fed Lactose-containing Diets.

Dietary group	Lactose content, %	No. of animals	Initial body wt, g	Gain in body wt over 40 day period,* g	% surviving†	Ovarian wt,* mg
A_1	0.0	8	44.5	114.6 ± 5.5 (8)	100	39.0 ± 2.9
B_1	0.0	8	44.4	129.0 ± 7.0 (8)	100	48.7 ± 5.3
A_2	30.2	8	45.6	96.5 ± 3.2 (8)	100	23.5 ± 3.7
B_2	30.2	8	44.3	119.2 ± 5.5 (8)	100	41.0 ± 5.2
A_3	45.3	8	44.6	84.3 ± 4.1 (8)	100	16.1 ± 2.7
B_3	45.3	8	44.5	107.4 ± 6.0 (8)	100	36.6 ± 3.9
A_4	60.5	8	44.9	44.0 ± 7.1 (2)	25	12.2 ± 1.4
B_4	60.5	8	44.7	82.6 ± 2.8 (7)	87.5	17.4 ± 1.8

The values in parentheses indicate the number of animals which survived and on which averages are based.

* Including standard error of the mean calculated as follows: $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

† Experimental period—40 days.

(Table II). Growth retardation was most marked in animals fed diet A_4 and B_4 (60.5% lactose) and was progressively less marked in animals fed smaller levels of lactose (diet A_3 and B_3, diet A_2 and B_2). At all levels of lactose feeding animals fed the whole liver powder gained significantly more weight than those fed a similar diet with liver omitted. This was particularly true for animals on the 60.5 per cent lactose ration. In the absence of dietary lactose, rats fed the whole liver powder (diet B_1) also gained more weight than animals on the liver free ration (diet A_1). The differences in growth between the latter, however, were not statistically significant. All lactose fed rats developed diarrhea during the first 10 days of the experiment. It occurred earlier and was more extensive in rats fed the higher lactose diets than in animals fed the 30.2% lactose diet. It was particularly marked on diet A_4. Subsequent to this period the diarrhea decreased in severity and gradually disappeared although it persisted in 2 rats on diet B_4 and 1 rat on diet B_3 for the duration of the experiment. In general no significant differences were noted between diet A and diet B in the incidence of diarrhea or its extensiveness for animals fed similar levels of lactose. For the first 10 days of feeding rats fed diets B_2, B_3 and B_4 did not differ in body weight from corresponding animals on diet A, although animals fed diet B_1 gained approximately 20% more weight than rats on diet A_1. Subsequent to this period, however, animals fed diets B_2, B_3 and B_4 consistently gained more weight than did corresponding rats on diet A. Two rats each on diets A_3 and A_4 developed extensive alopecia during the third week of the experiment. Alopecia was not observed in other rats on these diets nor in any of the animals fed liver-containing diets. Subsequent to the 4th week of feeding new hair replaced the areas of alopecia in rats fed diet A_3. The 2 rats with alopecia on diet A_4 succumbed. Six of the 8 rats on diet A_4 died during the experimental period with an average survival time for the decedents of 21.8 days (range 11-26 days). In contrast to the above only 1 rat on diet B_4 succumbed (8th day). All other rats survived. At autopsy a marked distension was observed in the cecum of rats fed diets containing 45.3

or 60.5% lactose. This was observed on both diets A and B. Cecal distension was also present on diets A_2 and B_2 but was less pronounced.

A significant difference in ovarian weight was observed in lactose-fed rats on the various diets (Table II). Ovaries appeared immature both in weight and microscopic appearance in animals fed diets A_2, A_3, A_4 and B_4. No mature follicles or corpora lutea were present in any of these rats with the exception of 1 animal on diet A_2. Six of the 8 rats on diet B_2 and 5 animals on diet B_3, however, had ovaries which were indistinguishable grossly and in histological appearance from normal controls (diets A_1 and B_1). In these animals mature follicles were numerous and corpora lutea were present.

Previous findings(10,11) indicate that the failure of rats to survive on diets containing high levels of lactose was due, at least in part, to an inability to hydrolyze adequately large amounts of this disaccharide. Present findings suggest that the administration of defatted and desiccated whole liver powder increased the rats' ability to digest lactose, although a period of approximately 10 days elapsed before this effect occurred. No data are available concerning the factor or factors in liver responsible for the increased growth, survival and ovarian development on lactose-containing diets. Recent findings by Hartman *et al.* (12) suggest, however, that vitamin B_{12} may be responsible, at least in part, for these effects.

Summary. The oral administration of 10% defatted and desiccated whole liver powder increased significantly the gain in body weight of immature rats fed purified diets containing 30.2, 45.3 and 60.5% lactose. It prolonged the survival time of immature rats fed a purified diet containing 60.5 per cent lactose, and it counteracted the inhibition of ovarian development which occurred on purified rations containing 30.2 and 45.3% lactose.

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Received November 9, 1949. P.S.E.B.M., 1949, v72.

Effect of Protein Level and Inanition on Glutamine Content of Rat Tissues.* (17531)

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A study by Roine(1) suggested the importance of glutamine in protein synthesis in yeast and indicated the need for investigation of the glutamine content of blood and tissues of higher animals. Wiss(2) observed that the blood glutamine content of rats increased when the rats were placed on an inanition routine and decreased when protein was fed. These results implied that blood glutamine concentrations are governed to some extent by the level of protein ingested. This work was undertaken to relate various dietary factors to changes in glutamine concentration in animal tissues.

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* Published with the approval of the Director, Oklahoma Agricultural Experiment Station. Supported by a grant from the United States Public Health Service through the National Institutes of Health. Appreciation is expressed to Merck and Company, Rahway, N.J., for supplies of crystalline vitamins and to the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y., for pteroylglutamic acid.

1. Roine, Paavo, *Suomen Kemistilehti*, 1946, v19B, 113.

2. Wiss, O., *Helv. Physiol. et Pharmacol. Acta*, 1948, v6, C35.

TABLE I.
Composition of Rations Used for Rat Dietary Studies.

Ingredient*	1	2	3	4
Casein (crude)	0%	10%	20%	40%
Sucrose	91	81	71	51
Cottonseed oil	5	5	5	5
Salts†	4	4	4	4

* Each kilogram of ration was supplemented with the following vitamins: Thiamin, 4 mg; riboflavin, 6; pyridoxine, 3; calcium pantothenate, 20; nicotinic acid, 20; pteroylglutamic acid, 1; inositol, 20; p-amino benzoic acid, 20; choline chloride, 1 g. In addition vitamins A and D and alpha tocopherol were administered by dropper twice a week.

† Hegstead *et al.*, *J. Biol. Chem.*, 1941, v138, 460.

Experimental. Experimental animals were young adult male rats of the Sprague-Dawley strain. Animals were placed on a 10-day equilibration period on the control ration (Ration 3, Table I) before use. In all studies food and water were available *ad libitum.*

After the equilibration period, animals were placed on rations of varying protein levels (Table I) for a period of 10 days. During the inanition experiment the animals were allowed access only to water. At the end of the experimental periods, the animals were killed by exsanguination under light ether anesthesia, the tissues rapidly removed, frozen and stored at −15°C until analyzed. Plasma glutamine determinations, however, were made immediately.

Glutamine was determined by nesslerization of the ammonia liberated after incubation with beef kidney preparations, essentially according to the procedure of Archibald.(3) The preparation of the tissues for the enzymatic determination involved homogenization of the weighed sample with 15 ml of 0.04 N potassium cyanide solution, dilution of the blended mixture to 20 ml with the potassium cyanide solution, and centrifugation of the resulting mixture for 10 minutes at 1500 R.P.M. Two ml aliquots (one ml in the case of liver) of the supernatant liquid were taken for each determination.

Results. The effect of varying the protein level upon the glutamine content of rat tissues is presented in Table II. Each value represents the average of two separate experiments using a total of 12 animals for each treatment.

TABLE II.
Relation of Protein Level of Ration to the Glutamine Content of Rat Tissues.

	Glutamine (mg/100 g or ml)			
Tissue	Ration 1	2	3	4
Plasma	3.4	3.1	2.8	3.3
Liver	189	148	97	79
Lung	174	97	74	50
Kidney	258	135	119	87
Heart	484	273	212	143
Spleen	356	172	156	136
Muscle	214	148	178	150
Protein level ingested, %	0	10	20	40

TABLE III.
Effect of Inanition on Glutamine Content of Rat Tissues.

	Glutamine (mg/100 g or ml)				
Tissue	0 hr	12	25	49	95
Plasma	2.9	3.8	3.7	2.8	2.4
Liver	92	85	99	148	183
Lung	73	63	89	140	171
Kidney	117	122	146	200	259
Heart	212	184	219	335	418
Spleen	156	145	204	267	332
Muscle	189	162	213	246	339

The relation between the glutamine content of rat tissues and length of inanition is shown in Table III. After a 10-day equilibration period on the control ration (Ration 3, Table I), the animals were subjected to starvation for the indicated periods, sacrificed, and the tissues analyzed as before.

Discussion. These studies indicated that there was a definite relationship between the glutamine content of rat tissues and the level of protein in the ration consumed. As the protein level increased, all tissues showed a decrease in glutamine content. Muscle tissue was different than the others studied in that this decrease was not found when protein was increased above the ten percent level. In contrast to tissues generally, the plasma glutamine level declined less sharply and then rose again; at 40 percent protein intake, values similar to those of animals consuming the protein deficient ration were found.

In the tissues of rats which have been

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3. Archibald, R. M., *J. Biol. Chem.*, 1944, v154, 643.

on an inanition regimen there was a definite initial trend towards a decreased glutamine content with deprivation of food. This trend was reversed, however, after 24 hours; and after 95 hours starvation, values similar to those found in rats subsisting on a protein-free ration were attained. In contrast to the report of Wiss(2) plasma glutamine levels did not increase, in fact, there was a suggestion of somewhat decreased values at 95 hours.

Both of these observations suggest that glutamine may have a function in the catabolism of amino acids, regardless of whether the source is dietary or from the body tissues. Whether this function is a direct one involving the utilization of glutamic acid or more indirect with glutamine acting as a reservoir for the ammonia released by the deamination of amino acids is not known. Preliminary studies in these and other laboratories do not indicate a direct conversion of administered glutamic acid to glutamine, thus suggesting that this amide may not be too closely involved in the metabolism of the corresponding dicarboxylic amino acid.

Summary. The variation of glutamine content of rat tissues with protein level or period of inanition has been studied. Glutamine levels showed a marked increase as the protein level of the diet decreased. An increased glutamine level was also observed with inanition. These observations indicate the possible importance of glutamine in protein metabolism.

Received November 10, 1949. P.S.E.B.M., 1949, v72.

Conversion of Tryptophane to Nicotinic Acid by *Trichophyton equinum*.*

(17532)

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A strain of *Trichophyton equinum*, one of the faviform trichophytons which commonly causes ringworm in horses, was found to require nicotinic acid.(1) *T. equinum* (Gedoelst) is a member of the group of large-spored, ectothrix trichophytons of animal origin which may infect man. They are characterized by their poor growth on the usual sugar-peptone media on which they produce slow-growing, glabrous, or slightly downy colonies composed largely of irregular hyphae and chlamydospores. By the use of natural carbohydrate media, Langeron and Milochevitch(2) and later Lebasque(3) showed that the growth and production of spores by these strains could be stimulated by better nutrition, and it was later shown(4,5,1) that many of the species in this group required certain vitamins and other growth factors present in natural substances. The requirement in most instances has been for thiamine and inositol, and in one case for pyridoxine as well. This Baudet strain of *T. equinum* obtained from the Culture Bureau for Fungi, Delft, Holland is the only strain known to show a requirement for nicotinic acid.

Experimental. No growth of this strain could be obtained on a vitamin-free basal agar prepared as follows: 50 g dextrose (C.P.), 2.0 g NH_4NO_3 (C.P.), and 0.5 g $MgSO_4$ (C.P.) dissolved in one liter of distilled water buffered to pH 7 with Sorenson's phosphate

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* This study was made with the help of a grant-in-aid from the United States Public Health Service.

† Present address: Communicable Disease Center, Mycology Laboratory, Chamblee, Ga.

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2. Langeron, M., and Milochevitch, S., *Ann. de parasitol.*, 1930, v8, 465.

3. Lebasque, J., *Ann. de parasitol.*, 1934, v12, 418.

4. Robbins, W. J., Mackinnon, J. E., and Ma, R., *Bull. Torrey Club*, 1942, v69, 509.

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mixture (KH_2PO_4 and Na_2HPO_4). To this is added 1.5% purified agar, prepared according to the method of Robbins.(6) Addition of nicotinic acid to such a medium allows rapid growth of the fungus. Nicotinic acid may be substituted for by equal amounts of nicotinic amide, and by comparatively large amounts of the amino acid 1-tryptophane.

All vitamins and amino acids were prepared in water solutions, sterilized by filtration, and added to the melted, partially cooled basal agar. Except for nicotinic acid and its amide, none of the other vitamins tested: thiamine, pyridoxine, para-amino-benzoic acid, i-inositol, choline, pantothenate, and folic acid showed any stimulatory effect when added singly or in combinations to the basal agar. The vitamins were added in amounts of 0.1 mg to 5 ml of the basal agar except for biotin where 0.05 mg was added. Twenty-two amino acids: glycine, dl-alpha alanine, beta alanine, dl-serine, dl-threonine, dl-valine, l-leucine, dl-norleucine, dl-aspartic acid, l-glutamic acid, l-lysine, l-arginine, l-histidine, l-cystine, dl-cysteine, dl-methionine, l-proline, dl-tryptophane, l-tyrosine, dl-phenylalanine, and l-hydroxyproline were tested singly and in combination in M/1000 dilution in the basal agar (with the exception of cystine which is highly insoluble, and was tested in more dilute solution). Of these only tryptophane showed any stimulatory effect.

The minimum effective concentration of nicotinic acid or amide was determined by diluting these substances serially (by halving) in tubes containing 5 ml of NH_4NO_3 basal broth (prepared similarly to the NH_4NO_3 basal agar described above except that no agar is added) and inoculating with washed fragments of a culture of *Trichophyton equinum* grown in a 1.0% peptone nutrient broth. Both nicotinic acid and nicotinic amide were active to a dilution containing 0.25 μg in 5 ml NH_4NO_3 broth. No growth occurred in higher dilution or in control tubes which contained no vitamin. By a similar titration it was determined that l-tryptophane was active to a dilution containing 625 μg in 5 ml NH_4NO_3 broth. dl-tryptophane showed only half this activity indicating that only the l form was active.

This substitution of the amino acid for nicotinic acid suggests the possibility of tryptophane as a precursor of this vitamin. In view of these findings, it was of interest to determine whether the strain of *T. equinum,* a naturally occurring nicotinicless fungus, could be shown to have the ability to convert tryptophane to nicotinic acid.

A series of 150 ml Erlenmeyer flasks each containing 50 ml NH_4NO_3 broth (described above), and a similar series containing 50 ml of the basal broth prepared without NH_4NO_3 were inoculated with small fragments of the fungus which had been grown for 7 generations on slants containing 1000 μg l-tryptophane in 5 ml NH_4NO_3 basal agar. Varying amounts of l-tryptophane were added to the flasks in duplicate series. One series was autoclaved immediately and served as control. The fungus grew in all of the non-autoclaved flasks containing tryptophane, but no growth occurred in control flasks of NH_4NO_3 basal broth. The amount of growth was roughly proportional to the amount of tryptophane present although in flasks where the NH_4NO_3 had been omitted and tryptophane was the only source of nitrogen, the amount of growth was much less.

After 6 weeks the entire growth and culture medium of each flask was hydrolyzed and biologically assayed for nicotinic acid with the *Lactobacillus casei.*‡ The results indicated

TABLE I.

Media*	Nicotinic acid assay‡	
50 ml basal broth +	Tests/μg	Controls/μg†
25 mg l-tryptophane	2.32	—
5 mg l-tryptophane	2.02	.0085
5 mg l-tryptophane + 100 mg NH_4NO_3	1.18	.008

*Basal broth—5% dextrose (C.P.) + 0.01% $MgSO_4$ (C.P.) in H_2O solution of M/15 phosphate buffers (KH_2PO_4 and Na_2HPO_4) at pH 7.

All flasks inoculated with *T. equinum* and maintained at room temperature 6 weeks before testing for nicotinic acid.

† Controls—media autoclaved immediately following inoculation with *T. equinum.*

‡ Nicotinic acid—determined by biological assay with *L. casei.*

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6. Robbins, W. J., *Am. J. Bot.*, 1939, v26, 772.

‡ The biological assay was very kindly performed by Dr. Ray F. Dawson of the Botany Department of Columbia University.

that nicotinic acid had been produced by the fungus in flasks containing tryptophane. The similar flasks which had been autoclaved immediately after inoculation with the fungus showed only negligible traces of nicotinic acid. (Table I). It is interesting to note that although twice to 3 times as much growth occurred in the tryptophane flasks which also contained NH_4NO_3, less nicotinic acid could be detected in these flasks. It seems possible that in the presence of readily available nitrogen, much of the nicotinic acid produced from the tryptophane was utilized as readily as it was formed.

Discussion. Krehl *et al.*(7) in their studies of the pellagragenic effect of corn were the first to show a possible interchangeable role of nicotinic acid and tryptophane. Later experiments by Rosen *et al.*(8) and Sarett and Goldsmith(9) indicated that tryptophane may be an important precursor of nicotinic acid in rats as well as in humans, and may explain the anti-pellagragenic effect of certain foods such as milk which are low in nicotinic acid but rich in protein. Bonner and Beadle(10) have described 3 x-ray mutants of *Neurospora* which require nicotinic acid for growth, and in a later communication, Beadle, Mitchell, and Nyc(11) have shown that one of the nicotinicless mutant strains, No. 65001, was able to grow when supplied with either tryptophane or nicotinic acid. By means of another nicotinicless mutant strain which could not utilize tryptophane, they further showed that strain No. 65001 actually had the ability to convert tryptophane to nicotinic acid. Nicotinic acid could also be produced by this strain from dl-kynurenine, a substance which has been known as a product of tryptophane metabolism in animals and bacteria, and is a possible intermediate in the conversion of tryptophane to nicotinic acid. On the basis of the growth responses of this *Neurospora* mutant strain to tryptophane, nicotinic acid, and kynurenine, it was postulated that nicotinic acid is normally formed from tryptophane with kynurenine as an intermediate.

According to Schopfer,(12) no naturally occurring fungi have been shown to require nicotinic acid. Whether the strain studied is a normally occurring type or an unusual mutant form is not known, and it is doubtful that the requirement for nicotinic acid by this strain of *T. equinum* is a characteristic which would be of value in species determination. The only other strain of *T. equinum* available for testing was No. 9870 from the American Type Culture Collection. This strain did not require nicotinic acid, but did require thiamine and i-inositol. In this respect it resembles *T. faviforme,* a common agent of ringworm in both cattle and horses. Since the separation of *T. equinum* from *T. faviforme* depends on the insecure grounds of its more frequent isolation from horses than from cattle and its ability to produce more aerial mycelium on the surface of colonies on Sabouraud's dextrose agar, it seems highly probable that *T. equinum* is synonymous with *T. faviforme.* Further strains must be obtained and studied in order to determine whether the requirement for nicotinic acid is present in any other strain of this group.

The transformation of tryptophane to nicotinic acid by the strain *T. equinum* (Gedoelst) gives further evidence for the hypothesis that, in both plants and animals, tryptophane is a precursor in the formation of nicotinic acid, essential to the growth of these forms.

Summary. A strain of *Trichophyton equinum* has been shown to have a requirement for nicotinic acid. Nicotinic acid may be substituted for by equal amounts of nicotinic amide and comparatively large amounts of l-tryptophane. The fungus has been shown to have the ability to convert tryptophane to nicotinic acid, thus giving further evidence in support of the hypothesis that tryptophane may be a precursor of nicotinic acid.

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Received August 3, 1949. P.S.E.B.M., 1949, v72.

Prodigiosin. I. Antibiotic Action on *Coccidioides immitis in Vitro*.* (17533)

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Prodigiosin is a thermostable, water insoluble, red pigment produced by *Serratia marcescens* under certain conditions. The dye has stimulated interest because of its ability as a contaminant to discolor water and food. This characteristic is better known than its inhibitory action against microbial agents.

The antibiotic properties of prodigiosin recorded in the literature indicate a possible wide spectral range of activity against bacteria and protozoa. No evidence concerning its fungistatic action could be found. Hettche(1) first reported the bacteriostatic activity of prodigiosin for staphylococci and *Bacillus anthracis in vitro*. The growth of *B. anthracis* was inhibited in a dilution of 1:200,000 after 3 hours' exposure and a dilution of 1:25,000 appeared effective against staphylococci after one hour's exposure. He was unable to demonstrate any activity against the gram-negative organisms, *Escherichia coli* and *Salmonella paratyphi*. Fischl(2) demonstrated that prodigiosin would protect mice against nagana. A single subcutaneous injection of 10 mg of prodigiosin in sweet almond oil caused the disappearance of *Trypanosoma brucei* from the peripheral blood.

Eisler and Jacobsohn(3) demonstrated that sterile boullion extracts of *S. marcescens* inhibited the growth of *Corynebacterium diphtheriae* and of *Neisseria gonorrhoeae*. Their results, however, were not directly related to dye content. Singh(4) reported toxic effects of the pigment of *S. marcescens* on soil amoeba. The concentration of the pigment which prevented amoebae from consuming an edible substrate of bacteria was not reported.

The naturally occurring dye was first isolated by Kraft,(5) who called it "prodigiosine." Wrede, Hettche, and Rothhaas(6) presented a series of articles on the chemical properties of prodiogiosin and identified the structure as a tripyrrole methane.

Materials and methods. Abundant pigment production was obtained from growth of *S. marcescens* on thick mannite agar plates (15.0 cm) adjusted to pH 5.0.† Surface inoculation with a single strain of *S. marcescens* (Stanford Z-4 strain) was followed by incubation at 22°C for from 5 to 7 days. The surface growth was harvested by scraping and contained large amounts of both intracellular and extracellular red pigment which was extracted by a modification of Wrede's method (Lack and Botts).(7) Yields of from 1.0 to

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* Sponsored by the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are a result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

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5. Kraft, E., Thesis Wurzburg, 1902 (from Wrede, F., and Hettche, O., *Berichte d. Deutschen Chemischen Gesellschaft*, v929, v22, II Abblg. B, 2678).
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†Mannite agar formula:

Mannite	20 g
Neopeptone	10 g
Meat extract	3 g
$MgSO_4$	1.25 g
Brewer's yeast	1 g
Agar	20 g
Q.S. ad H_2O	1000 cc

Adjusted to PH 5.

7. Lack, A., and Botts, E., in press.

TABLE I.
Fungistatic and Fungicidal Activity of Prodigiosin Base and Certain Salts on *Coccidioides Immitis in vitro.*

Derivative	Fungistatic conc., ×1000	Fungicidal conc., ×1000
Base, purified	1:500	1:100
Hydrochloride	1:500	1:100
Perchlorate	1:500	1:100
Glutamic acid (water soluble)	1:250	1:83
Tartrate	1:100	1:50
Sulfonate	1:10	0
Sulfonamide	1:1	0

2.0 mg of prodigiosin were obtained from approximately 10 sq cm of wet surface growth. Repeated purifications yielded a prodigiosin from which several new compounds were made.[‡] Each compound was tested *in vitro* for its fungistatic and fungicidal action against *Coccidioides immitis* (Strain S.F.S. 46). Inasmuch as prodigiosin base is insoluble in water, tests were made to determine the most satisfactory solvent. Propylene glycol and ethanol were found to be suitable vehicles for *in vitro* and *in vivo* tests. Propylene glycol is sufficiently miscible in water to permit uniform dilutions of the derivatives to be tested in liquid synthetic culture medium.

Serial dilutions of prodigiosin in propylene glycol or ethanol from 1:10,000 to 1:10,000,000 were made in a synthetic medium.[§] Controls with equivalent amounts of propylene glycol or ethanol in this medium were run in parallel. Each tube was inoculated with a 0.1 ml of a known concentration of chlamydospores which was diluted to give a range of from 2.0 to 3.0×10^4 spores. After incubation of 24 and 48 hours at 34°C 0.1 ml was transferred from each tube to 9.9 ml of the liquid synthetic medium as a test for viability. The subcultures were incubated at 34°C and inspected for growth after from 10 to 14 days on a basis of no growth (0) to abundant growth (++), indicating first signs of spore germination to rich mycelial development.

Results. The prodigiosin first prepared was fungistatic in a dilution of 1:250,000. Subsequent purifications increased the activity to 1:500,000 (Table I). Fungicidal activity as determined by the subcultures showed no growth at 1:100,000 dilutions. Propylene glycol controls revealed no inhibition of *C. immitis* except in the first tube of the series containing 10% propylene glycol. The hydrochloride and perchlorate salts of prodigiosin base inhibited growth in a dilution of 1:500,000. The tartrate maintained moderate effectiveness; the sulfonate and sulfonamide derivatives showed an almost complete loss of activity. A water soluble preparation was made by fusion of glutamic acid with prodigiosin base. This glutamic acid compound demonstrated effective fungistatic activity at a 1:250,000 dilution.

A comparison was made of the fungistatic and fungicidal effectiveness of prodigiosin purified bases when dissolved in propylene glycol or ethanol. Inhibition of growth of *C. immitis* was obtained in a dilution of 1:500,000 in both series. Although definite inhibition occurred at a dilution of 1:1,000,000, after several weeks' incubation germination appeared first in the propylene glycol and then the alcohol series. The propylene glycol and alcohol controls showed abundant growth in all except the initial tubes containing equivalent amounts of diluent. The tubes were subcultured at 48 hours to test the fungicidal activity. Slightly better results were obtained

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[‡] The preliminary investigation of the chemical constitution and general properties of prodigiosin were performed with the assistance of Dr. Frederick Proescher and Mr. V. Sycheff, formerly of the Santa Clara County Hospital laboratory. Subsequent extension of similar chemical research has been with the aid of Dr. Elbert Botts in the laboratory of the Birmingham Veterans Administration Hospital, Van Nuys, Calif.

[§] Synthetic media:

	g
NH_4Cl	10
Na Acetate	10
KH_2PO_4	2
K_2HPO_4	6

H_2O q.s. ad 1000 cc pH 6.12.

in the propylene glycol series. Complete loss of viability of all chlamydospores after 48 hours' exposure was established at a prodigiosin dilution of 1:100,000.

Summary. Prodigiosin was fungistatic for *Coccidioides immitis* in dilutions through 1:500,000. It was fungicidal after 48 hours' exposure in dilutions through 1:100,000. Prodigiosin hydrochloride, perchlorate and a water soluble glutamic acid derivative demonstrated similar *in vitro* effectiveness.

Received October 14, 1949. P.S.E.B.M., 1949, v72.

Pregnancy in Alloxan Diabetic Rats. (17534)

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Because of the well recognized frequency of complications in the course of pregnancy in clinical diabetes, this study was undertaken to determine the course of pregnancy in rats suffering from uncontrolled chronic diabetes. Since August 1946, when these studies were begun, 4 reports(1-4) have appeared dealing with this subject each with a slightly different approach to the problem and each with somewhat varying results.

Methods. Twenty-seven 70-90 day old Wistar strain female rats were fasted overnight and given a single subcutaneous dose of alloxan varying from 140-175 mg per kg. Where a single dose failed to produce a persistent diabetic state, repeated injections of increasing doses were employed up to 205 mg per kg. In some rats it was necessary to administer as many as nine doses of alloxan in order to achieve permanent diabetes. In 2 rats permanent diabetes did not appear despite repeated injections of alloxan. During the entire period covered by these studies the animals were kept in individual metabolism cages and the following data were obtained daily, 7 days a week, for periods up to 448 days: (a) total fluid intake, (b) total food intake, (c) total urine volume, (d) total quantitative glycosuria, (e) qualitative acetonuria, (f) vaginal smears for determination of oestrus. The animals were given free access to a constant mixture of crushed Purina Lab Chow (Protein 25%, Carbohydrate 50%, Fat 6.3%) reinforced with the following: reduced iron 0.2 g; sodium chloride (iodine-free) 10 g; sodium chloride (iodized) 2.7 g; riboflavin 5 mg, vitamin B_6 5 mg, vitamin D 3000 U.S.P. units, vitamin E 100 mg, choline 15 mg, per kilo of chow in order to bring the ration up to an adequate level for breeding as recommended by the Ralston Purina Company.

Because of the frequent occurrence of a transient diabetic state after alloxan injection, matings were performed when possible only after the establishment of permanent diabetes by placing one young adult male of the same strain into the metabolism cage with each female on the day of oestrus. Animals were considered definitely diabetic only when they fulfilled both of the following criteria: (1) persistent glycosuria greater than 0.5 g per 24 hours as measured by Benedict's Picrate method, and (2) repeated non-fasting venous blood sugars in excess of 200 mg% obtained from tail blood or cardiac puncture and measured by the technique of Lauber and Mattice.(5) Fasting blood sugars were not employed in this study, because it was thought that repeated overnight fasts might introduce an irregular influence on the nutritional or

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1. Davis, M. E., Fugo, N. W., and Lawrence, K. G., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 638.

2. Miller, H. C., *Endocrinology*, 1947, v40, 251.

3. Hultquist, G., *Acta Path. et Microbiol. Scand.*, 1948, v25, 131.

4. Sinden, J. A., and Longwell, B. B., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 607.

5. Lauber, F. V., and Mattice, M. R., *J. Lab. and Clin. Med.*, 1944, v29, 113.

diabetic state of the animals. In those animals considered permanently diabetic, with two exceptions (c.f. Rats no. 18 and no. 19 below) the criteria for diabetes remained satisfied until the animals were either sacrificed, died, or were removed from the experiment after periods varying from 32 to 425 days.

Results. Of the 25 rats in which permanent diabetes was produced, 12 pregnancies were achieved in 7 rats, 2 of which had 3 pregnancies, 1 had 2 pregnancies and the remainder 1 each. Of the 12 non-diabetic control rats of the same age and strain as the experimental animals, 11 pregnancies were produced in 8 rats over the same period of time. Despite the absence of any effort at treatment of the diabetes, clinical acidosis was not observed in any of the 25 permanently diabetic rats. In no case was insulin administered. Nevertheless, most of the animals remained in apparent good health, with maximum 24 hour water intakes of 200-400 cc and maximum urine outputs of 70-140 cc. Occasional isolated instances of acetonuria were observed. When olive oil was added to the diet, however, acetonuria was frequent and persistent. No blood carbon dioxide studies were performed, but clinical evidence of acidosis failed to appear. Blood sugars in the control group taken under the same non-fasting conditions as in the diabetic group averaged 95 mg% with variation from 79 mg% to 119 mg%. Diabetic animals revealed blood sugars varying from 224 mg% to 650 mg%, with a single exception of one value of 133 mg%. The average of all diabetic blood sugars was 450 mg%.

As previously reported,(1,4) there was disappearance of oestrus vaginal smears for long periods of time and irregular oestrus cycles appeared in females with well established diabetes. In rats made diabetic by subtotal pancreatectomy Richter *et al.*(6) reported the return of normal oestrus activity, when these animals ingested a high fat diet. The main constituent of this high fat diet was olive oil. Richter's observations are in large measure confirmed in our studies. Animals partook of olive oil when it was offered and there was a definite trend toward normal oestrus cycles as a result thereof. In most cases, however, irregularities persisted and in no case did pregnancy occur after the use of olive oil.

Observation of these 12 diabetic and 10 normal pregnancies revealed the following data: (1) The estimated period of gestation varied between 20 and 29 days in the diabetic group and between 20 and 28 days in the control group, with the majority of both groups varying between 20 and 23 days. (2) No significant alteration in the severity of the diabetes was noted during pregnancy or in the puerperium in any of the animals, with the 2 exceptions, *c.f.* Rats no. 18 and no. 19 below. (3) Though urines were not tested routinely for albumin and no blood pressure studies were performed, there was no clinical evidence of toxemia and the pregnancies in the diabetic animals appeared quite as normal as those of the controls. (4) Figures for the number of young per litter as determined by observation were found to be unreliable both in the diabetic and control series, because of the tendency in both groups to some degree of cannibalism. In spite of this, however, no significant difference was noted in the number of offspring per litter in the two groups. (5) No external congenital anomalies were observed at birth in any litters in either the experimental or control series and, so far as clinical observation could determine, the fetuses were grossly of equal size in the two groups. Four offspring of 2 diabetic animals were observed to maturity and these animals proved to be completely normal in all respects. Repeated blood sugar levels were determined in these offspring of diabetic mothers after weaning at 28 to 35 days and no deviation of the blood sugar from normal was observed. (6) Because of the size of the newborn rat it was impossible to measure the blood sugar level and since a clinical diagnosis of hypoglycemia under these conditions was considered valueless, no data were obtained on this phase of the subject. (7) Compared with their prepregnant course and the diabetes of other nonpregnant rats, no alteration in the course of the disease was noted in 5 rats subsequent to

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6. Richter, C. P., Schmidt, E. C. H., Jr., and Malone, P. D., *Bull. Johns Hopkins Hosp.*, 1945, v76, 192.

TABLE I.
Data on Pregnant Diabetic Rats.

Rat No.	Duration of diabetes (days)	Alloxan		Glycosuria (non-pregnant period) avg*	Blood sugars (non-pregnant period) range, mg%	Duration of diabetes prior to pregnancy days	Glycosuria (pregnancy) avg*	Blood sugars (pregnancy) (mg%)	Avg
		Doses	mg/kg						
8	351	6	140	4.15 (0.19-12.6)†	330-598	(1) 27 (2) 100 (3) 145	(1) 2.99 (.12-6.7)† (2) 3.40 (.57-6.3) (3) 4.20 (.44-11.8)	(1) 290-432 (2) 400-580 (3) 432 (1)	392 480
16	425	8 1	140 165	0.95 (.00-12.6)	270-522	(1) 50 (2) 124 (3) 207	(1) 0.75 (.0-4.0) (2) 2.87 (.0-8.1) (3) 2.64 (.0-8.0)	(1) 224-400 (2) 380-488 (3) No blood sugar	368 434
17	405	5 2 1	140 180 200	2.85 (.00-5.3)	236-650	(1) 23 (2) 134	(1) 0.58 (.0-3.1) (2) 4.53 (.0-12.1)	(1) 340-400 (2) 402-480	375 471
18	68	3	140	1.28 (.00-2.5)	300-444	53	1.55 (.28‡-3.7)	368 and 26 (2)	
19	32	5 1 1	140 180 205	1.1 (.48-1.7)	250 (1)	4	1.02 (.0-4.0)	444 and 810 (2)	
23	130	1	170	2.56 (.82-5.2)	345-444	4	1.75 (.0-6.1)	328 and 133 (2)	
27	274	2	175	4.23 (1.2-11.6)	246-456	16	1.52 (.27-3)	380 single	

* g/24 hr. † Range. ‡ .0 on day of death. ‖ Day before delivering.

single or repeated pregnancies. (8) In Rats No. 18 and 19 of this series a phenomenon was observed which we are at a complete loss to explain. Both of these animals died in hypoglycemia. Neither of them was given insulin at any time. Rat No. 18 was first noted to be somewhat apathetic on the 15th day of her only pregnancy, 68 days after the onset of her diabetes, which prior thereto had not differed from that observed in our other animals. Blood sugar 13 days before death was 368 mg%. Glycosuria in excess of 0.5 g in 24 hours had persisted until the day before death. Shortly after the apathy appeared cyanosis developed. There was bleeding from the nose and vagina. The coat became ruffled and the temperature fell to 99° (R). Five hours after the onset of this train of symptoms the animal was moribund. A cardiac puncture revealed a blood sugar of 26 mg%. Death ensued within 15 minutes. Autopsy was performed immediately. There were no gross abnormalities. Microscopically, the kidneys showed colloid droplet degeneration in both the proximal and distal convoluted tubules. The sections of the eyes showed the presence of incipient cataracts. Well-formed islets of Langerhans were present in sections of the pancreas. There was no necrosis or excessive fat deposition in the adrenals or liver. Sections of the other organs including the pituitary were not remarkable.

Rat No. 19 died with identical symptoms 6 days after delivery of a litter of 9 living young, 32 days after the onset of her disease. Her diabetes had likewise run the usual course. Blood sugar 16 days prior to death was 810 mg%. Following delivery the animal ate no chow and drank little water but devoured her entire litter. She became moribund 8½ hours after the onset of symptoms. Cardiac blood sugar at this time was 22 mg%. Death occurred about 15 minutes later and autopsy was performed immediately. No gross abnormalities could be made out. Microscopically, the only positive finding was the presence of incipient cataracts in sections of the eyes.

Discussion. The results of this study of 12 diabetic and 10 control pregnancies confirm previous reports(1,4) that alloxan diabetes, like pancreatectomy diabetes(6) interferes with normal oestrus. Administration of olive oil to these animals tends to restore a more normal oestrus pattern as demonstrated by Richter(6) for subtotally pancreatectomized animals, but does not influence fertility.

Our results are contrary to those of Davis *et al.*(1) who reported the resorption of fetuses in 4 uncontrolled alloxan diabetic rats and stated that control of the hyperglycemia by insulin, as demonstrated in 7 treated rats, was essential to successful completion of pregnancy with live litters.

Miller(2) reported 10 normal pregnancies in 8 diabetic rats where the average glycosuria per day varied from 0.27 g to 0.5 g, in 3 untreated animals, and from 0.3 g to 1.6 g per day in 7 rats treated with protamine zinc insulin in order to prevent severe glycosuria. No blood sugars were reported. These untreated animals demonstrated mild diabetes as judged by our requirement of a glycosuria in excess of 0.5 g in 24 hours.

Davis *et al.*(1) reported that in untreated alloxan diabetic rats "pregnancy progressed normally until about the 12th day following which the fetus died and was slowly absorbed". Our data, however, do not support this finding and confirm those of Sinden and Longwell.(4) Eleven of the 12 pregnancies studied progressed normally to term with the delivery of normal litters. The need for insulin as stressed by Davis *et al.*(1) did not exist in this series. In fact, the only rat that died during pregnancy manifested a terminal hypoglycemia.

Summary and conclusions. (1) Alloxan diabetes, like pancreatectomy diabetes, interferes with the normal oestrous cycle of rats, and impairs fertility.

(2) Administration of olive oil aids in reversion of the oestrous cycle toward normal, but does not improve fertility.

(3) The course of 11 of the 12 pregnancies reported in this study was uninfluenced by the presence of pre-existing permanent alloxan diabetes.

(4) The course of the diabetes in 5 of the 7 rats observed was not influenced by single or repeated pregnancies.

(5) Two rats died in hypoglycemia, one on the 15th day of pregnancy and one 6 days

post partum. The mechanism of this hypoglycemia is unexplained.

(6) Administration of insulin was not required in order to obtain live, full term litters in 10 of the 11 pregnancies observed.

Received October 11, 1949. P.S.E.B.M., 1949, v72.

Purification of Certain Viruses by Use of Protamine Sulfate. (17535)

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During attempts to improve the immunogenic potency and to increase the purity of Japanese encephalitis vaccine, it was noted that the addition of protamine sulfate to suspensions of infected chick embryo tissue produced a heavy precipitate, and, of particular interest, left almost all of the virus in the clear supernatant fluid. Subsequently, a number of viruses were studied with respect to their behavior in the presence of protamine. It was found that they fell into 2 groups, *i.e.*, those which were precipitated along with the tissue materials as has been observed by others(1,2) and those, like Japanese encephalitis virus, which remained in the supernatant fluid. For this latter group, protamine precipitation provides a rapid, simple and effective means for partial purification.

Methods. A considerable number of orienting experiments indicated that protamine precipitation could be carried out in the following manner: A chilled infected 10% suspension of mouse brain clarified by low-speed centrifugation or a 20% suspension of uncentrifuged chick embryo tissue, pH 7.0 to 7.2, is added to protamine sulfate (salmine) in the proportion of 1.0 ml of tissue suspension for each 5.0 mg of protamine. Either the readily soluble dry protamine powder or a solution of the material is used. A fine precipitate forms immediately on the addition of the tissue suspension to the protamine. The mixture is stored with occasional agitation in the refrigerator for 30 minutes after which the precipitate is separated by low-speed centrifugation at 3000 RPM for 15 minutes in an angle centrifuge. The resultant clear, pink supernatant fluid is decanted and saved; in the present report it is designated as "protamine supernate." Protamine supernates obtained by the method just described develop a granular precipitate during the first 24 hours of storage in the refrigerator. Furthermore, if formaldehyde is added in order to inactivate the virus, a heavy flocculation slowly develops. This flocculation apparently is due to the residual protamine in the preparations. It can be avoided if the excess protamine is removed by precipitating it with heparin. The optimum amount of heparin for this purpose is 3.8 mg per ml of supernate. Less than this amount fails to remove sufficient protamine whereas more produces a finely dispersed precipitate which sediments with difficulty. Thirteen viral agents were subjected to treatment with protamine. Their distribution in the various fractions thus obtained was determined by assaying the infectivity of the materials. In all but 2 instances this was done by intracerebral titration in mice in the usual manner. The exceptions were mumps virus, which was assayed in embryonated eggs by testing for the presence of viral hemagglutinins in the chorioallantoic fluids, and one strain of vaccinia virus, which was titrated on the chorioallantoic membrane.

Results. Action of protamine on infected suspensions. The viral agents which remain in the clarified supernatant fluid after treatment with protamine are listed in Table I, as are those which are precipitated along with tissue materials by this treatment. Others

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1. Chambers, L. A., and Henle, W., PROC. SOC. EXP. BIOL. AND MED., 1941, v48, 481.

2. Bawden, F. C., and Pirie, N. W., *Proc. Roy. Soc., Series B*, 1937, v123, 274.

TABLE I.
Infective Titer of Crude and Protamine-treated Suspensions of Virus Infected Tissues.

Virus	Source	Infective titer		
		Crude suspension	Protamine-treated Supernate	Sediment
	Agents not precipitated			
Colorado tick fever	Mouse brain	6.2	5.2	3.8
Equine encephalomyelitis (western type)	” ”	8.4	8.2	N.D.
Encephalomyocarditis	” ”	8.5	8.5	N.D.
Japanese encephalitis	” ”	8.4	8.4	<6.0
	Chick embryo	7.3	7.2	<6.0
Poliomyelitis (Lansing)	Mouse brain	3.4	3.2	N.D.
	Cotton rat brain	3.0	3.0	N.D.
Russian Spring-Summer encephalitis	Mouse brain	8.7	8.5	N.D.
St. Louis encephalitis	” ”	6.7	6.8	N.D.
West Nile virus	” ”	7.6	7.6	N.D.
	Agents precipitated			
Herpes	Mouse brain	4.3	<1.5	3.8
Lymphocytic choriomeningitis	” ”	5.2	<2.5	5.2
Murine encephalomyelitis (GD VII)	” ”	7.4	<4.0	6.8
Rabies	” ”	6.6	3.5	5.4
Vaccinia	” ”	5.2	<3.0	N.D.
	Chick embryo	8.2	4.5	7.0

have shown that influenza(1) and tobacco mosaic(2) viruses are precipitated by protamine. Most of the observations on factors influencing the protamine reaction were made on suspensions of chick and mouse tissues infected with Japanese encephalitis virus. The amount of protein nitrogen, *i.e.*, acetic acid precipitable N, in the mouse brain protamine supernatant was about one-third that in the crude tissue suspension. When the protamine precipitation was performed at various temperatures ranging from 2°C to 30°C, there was little variation in the virus titer and total nitrogen in the protamine supernates. However, when the temperature was over 30°C, the supernates were never quite clear. We have chosen to perform the procedure in the cold to maintain maximum infectivity of the viral materials. Other observations indicated that the technic was equally effective when carried out over the pH range from 6.0 to 8.0.

Purity of protamine clarified materials. On the basis of infectivity and total nitrogen values the purity of preparations of Japanese encephalitis virus obtained by ultracentrifugation of protamine supernatants was appreciably greater than that of viral suspensions obtained by the usual differential centrifugation procedures. In 2 experiments aliquots of a suspension of mouse brain were treated by the 2 methods. Protamine supernate prepared from 1 aliquot was centrifuged at 100,000 G for 1 hour after which the sediment was resuspended and saved. The other aliquot was first clarified by centrifugation at 10,000 G for 1 hour and then subjected to the same treatment as the protamine supernate. The total nitrogen content of the ultrasediments, expressed as gamma/ml of suspension subjected to ultracentrifugation, was 7.0 and 3.0, respectively, for the 2 protamine-treated materials, and 73 and 40, respectively, for the materials concentrated by centrifugation alone. It should be emphasized that in one experiment the infective titers of the original suspension and of the resuspended sediments obtained by both procedures were of the same order of magnitude, *i.e.*, $10^{-7.0}$-$10^{-8.0}$.

Electron micrographs were made of preparations of Japanese encephalitis virus obtained from infected mouse brains by combined treatment with protamine and heparin followed by ultracentrifugation. These showed little other than numerous spherical particles with a diameter of approximately 20 mμ; the size of

TABLE II.
Comparative Potencies of Crude and Purified Japanese Encephalitis Vaccines (10% Mouse Brain).

Vaccine No.	Purification	Minimal immunogenic dose, ml
185A	Crude	.001
185A	Protamine*	.008
172	Protamine*	.007
176	Crude	.006
176	Protamine-heparin	.007
173	” ”	.005
180B	” ”	.002
181	Protamine-heparin + Ultracentrifugation	.004

* Vaccines inactivated by ultraviolet light. All other vaccines inactivated with formaldehyde.

Japanese encephalitis virus is about 15-22 mμ. Similar particles were also found in sediments from suspensions of normal mouse brain prepared in the same manner. Hence, it was not possible to differentiate between the Japanese encephalitis virus and particles of the sedimentable component which were present in normal mouse brain preparations.

Antigenicity of protamine-treated Japanese encephalitis vaccines. Japanese encephalitis vaccines were prepared from infected mouse brain and partially purified by treatment with (a) protamine, (b) protamine and heparin, or (c) by ultracentrifugation of protamine-heparin treated materials. In most of these the virus was killed by 0.2% formaldehyde. Two lots were inactivated by irradiation with ultraviolet light. The data summarized in Table II indicate that each of these preparations when assayed in mice would meet the potency requirements established for Japanese encephalitis vaccine.(3)

One of the lots of protamine-heparin treated vaccine (180B, Table II) was tested for its immunogenic activity in human beings. Seven volunteers were given 0.1 ml of the purified material intradermally while 8 received 1.0 ml subcutaneously. All of these persons had previously received one or more courses of crude chick embryo type Japanese encephalitis vaccine. The level of Japanese encephalitis neutralizing antibody before and after injection was determined by the intracerebral mouse test. Five of the volunteers had detectable amounts of specific antibody before receiving the present vaccine (neutralization indices between 100 and 650). All 5 of these showed a marked rise in titer following their booster dose with indices which now ranged between 2500 and 40,000. In addition, 6 of the 10 volunteers without detectable antibody prior to vaccination subsequently developed significant amounts of antibody with indices ranging from 100 to 15,000. The antibody response was similar in the groups who received vaccine intradermally and subcutaneously, and did not differ appreciably from that previously noted in persons receiving booster doses of the standard Japanese encephalitis vaccine by the same routes.(3)

It may be mentioned that vaccine 180B had a titer of 1:16 when used as antigen in a complement-fixation test performed according to the technic previously employed in this laboratory.(3) This is within the limits generally obtained for antigens prepared from mouse brain infected with this virus. Protamine-treated antigens of a different type have been used by Roberts(4) in a serological flocculation test for poliomyelitis.

Summary. Suspensions of a number of viral agents can be partially purified by treatment with protamine sulfate which precipitates much of the extraneous material leaving essentially all of the virus in the clarified fluid. Preparations obtained by this simple method have potential value as antigens, vaccines and starting material for studies on the physical and biochemical nature of selected viral agents.

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3. Warren, J., Smadel, J. E., and Rasmussen, A. F., *J. Immunol.*, 1948, v58, 211.

4. Roberts, E. C., *Pub. Health Rep.*, 1949, v64, 212.

Received November 16, 1949. P.S.E.B.M., 1949, v72.

Cytotoxic Action of Antisera to Cell Components of Normal and Leukemic Mouse Spleens.* (17536)

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Serologic studies, employing cytoplasmic fractions segregated from the spleens of normal and leukemic mice(1) demonstrated quantitative, but not qualitative, differences. Since nitrogen values had been made the basis for dilution of all fractions for immunization purposes, as well as for use in serologic tests, we postulated that some other antigenic component—not measurable entirely by nitrogen evaluation—could account for the quantitative differences, as expressed by the consistently higher serum titers for the leukemic fractions. The experiment was repeated with a second lot of cytoplasmic fractions, prepared in Dr. Mary L. Petermann's laboratory, and described as follows:

"The mitochondria were separated and washed in 0.88 M sucrose as in earlier experiments,(2) except that cycles of low and high speed centrifugation were employed, to insure the removal of larger as well as smaller particles. The two fractions of submicroscopic particles, P_1 and P_2, were isolated from the mitochondria supernatant by centrifuging for two hours at 180,000 x gravity. The P_1 fraction came from the pellets, while the P_2 came from the layer just overlying the pellets. Each fraction was washed once with 0.44 M sucrose. A detailed description of these particles will be published elsewhere."(3)

These cell preparations were used by us for immunization of rabbits, and as antigens in complement fixation tests. In all details these procedures followed those of the earlier study. Likewise, the results reflected those of the first experiment. Further study demonstrated that these spleen antisera—both normal and leukemic—are injurious to leukemic cells upon *in vitro* exposure. Moreover, a marked difference, as measured by survival rates of mice receiving serum-treated cells, appears between the cytotoxic effects produced by the normal and leukemic antisera in suitable dilutions, differences far greater than those revealed by complement fixation procedures.

While cytotoxic properties of antisera to various tumors have been reported by Lumsden,(4) Kidd,(5) Andervort,(6) Green,(7,8) Burmeister,(9) such action of antisera on the leukemic cell has not been described.

In this laboratory however Stock§ observed that exposure of leukemic cells to anti-leukemic cell (whole cell) or anti-normal mouse tissue sera, prior to injection of a small series of mice, resulted in a high percentage of survivals. Undiluted sera were used and the survival rate in the two groups of animals was very similar.

Materials and methods. Antisera. The following antisera to spleen components were tested for cytotoxic action:

1) Mitochondria, normal and leukemic
2) P_1, normal and leukemic

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* This study was supported by grants from the Office of Naval Research and the James Foundation of New York, Inc.

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1. Dulaney, Anna Dean, Goldsmith, Yvette, Arnesen, Kristen, and Buxton, Louise, *Cancer Research*, 1949, v9, 217.

2. Alfin-Slater, R. B., Larack, A. M., and Petermann, M. L., *Cancer Research*, 1949, v9, 215.

3. Petermann, M. L., Larack, A. M., and Handler, R. S., in preparation.

4. Lumsden, Thomas, *Am. J. Cancer*, 1937, v31, 430.

5. Kidd, John G., *Science*, 1944, v99, 348.

6. Andervort, Howard B., and Bryan, W. Roy, *J. Nat. Cancer Inst.*, 1944-45, v5, 143.

7. Green, Robert G., PROC. SOC. EXP. BIOL. AND MED., 1946, v61, 113.

8. Green, Robert G., *Science*, 1947, v107, 93.

9. Burmeister, B. R., *Cancer Research*, 1947, v7, 459.

§ Personal communication, Dr. C. Chester Stock.

3) P_2, leukemic[||]
4) Nuclei, normal and leukemic(10)
5) Whole cell, normal and leukemic
6) Heterologous tissue (liver, lung, kidney)

As previously stated, all preparations were standardized on the basis of nitrogen content. All rabbits received 6 doses of antigen adjusted to contain the same amount of nitrogen.

The source of the leukemic cells used in Tests. The strain of lymphatic leukemic (No. 9421) was carried routinely by intraperitoneal injection of inbred mice (brother-sister matings) with leukemic cells. The donor spleen was minced finely in 19 parts of physiological saline solution and the cells filtered through cotton by aspiration into a tuberculin syringe carrying a 26 gauge needle. The number of cells per 0.1 ml was determined by counting in a haemocytometer. Following intraperitoneal inoculation with 1,000,000 cells, mice consistently died after 10-12 days and upon autopsy exhibited typical leukemic spleens with an average weight of 0.4-0.6 g. The spleens of these inbred mice were used as sources of leukemic cells for all the experiments reported here. Akm mice were however used as test animals.

Tests for Cytotoxic Action of Antisera. The test dose for all the cytotoxic tests and controls consisted of 100,000 cells introduced intraperitoneally. All antisera had been heated for 30 minutes at 56°C prior to use. Measured amounts of antisera, in the dilutions indicated, were combined with leukemic cells. Guinea pig complement[¶] was added in the proportion of 0.1 ml of complement to 1.0 ml of antiserum and cells,[**] and the mixture incubated at 37°C for 2 hours with frequent stirring.

Controls. The following controls were included: 1) Cells + physiological saline solution, injected without preliminary incubation. 2) Cells + physiological saline solution + complement, incubated for 2 hr at 37°C. 3) Cells + normal rabbit serum + complement.

One series of mice received this mixture immediately while a second series of animals received identical doses after incubation at 37°C for 2 hours. All mice were observed daily and each mouse dying after the 10th day was autopsied and the size of the spleen recorded. At intervals spleens from certain of the groups were combined and the average weight recorded.

Results. Cytotoxic Tests. Table I shows the survival rates among 352 mice serving as controls. One of 96 mice receiving the dose of 100,000 leukemic cells immediately after standardization survived. Incubation of the same dose of leukemic cells with complement and physiological saline, or with normal rabbit serum and complement, produced a slight increase in the number of survivals. The differences in survival rates of mice receiving 100,000 leukemic cells in combination with undiluted antisera to normal and leukemic spleen fractions were not significant; therefore, a table is omitted. This confirms Stock's observation with the undiluted antisera to whole cells. There was, however, a significantly lower survival rate of mice receiving antisera to heterologous organs. The spleen lymphocyte thus stimulates the formation of a specific antibody effective against the leukemic cell. When the antisera to the normal and leukemic cell fractions were diluted in serial fashion, prior to combination with the leukemic cells, there was demonstrated a highly significant difference in the cytotoxic action. This suggests that the concentrated antibody of the undiluted sera may have masked the true effects, apparent upon dilution. Table II gives the complete data from titrations.

It is apparent that the mitochondria antisera exhibited the highest titers but the least difference between the normal and leukemic. The P_1 antisera, while of lower titers, exhibited the greatest differences in effects. Compari-

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[||] The yield of P_2 particles differed quantitatively and it was not possible to obtain sufficient material from the amount of normal spleen used.

10. Arnesen, Kristen, Goldsmith, Yvette, and Dulaney, Anna Dean, *Cancer Research*, 1949, v9, 669.

[¶] Carworth Farms—"Vacseal."

[**] It was not determined whether complement was necessary for the action of the serum. This subject is reviewed by Leymaster and Ward.(11) It was established however that complement did not interfere in any way with the phenomenon of cell injury.

11. Leymaster, Glen R., and Ward, Thomas G., *J. Immunol.*, 1949, v61, 95.

TABLE I.
Showing the Survivals in the Groups of Mice Serving as Controls. All Received Standard Doses of Leukemic Cells, but No Anti-Sera.

Combination	No. of mice	No. of survivals	% of survivals
Cells and saline; no incubation	96	1	1
Cells and saline and complement; incubated 2 hr	157	8	5
Cells and normal rabbit serum and complement; no incubation	49	2	4
Cells and normal rabbit serum and complement; incubated 2 hr	50	2	4

TABLE II.
Showing the Cytotoxic Action of Diluted Antisera on Leukemic Cells.

Antiserum	Dilution of serum	No. of mice	No. of survivals	% of survivals
Normal mitochondria	1:10	10	9	90
	1:100	10	10	100
	1:200	10	9	90
	1:500*	30	8	27
	1:750	20	3	15
Leukemic mitochondria	1:10	10	10	100
	1:100	10	9	90
	1:200	9	9	100
	1:500*	30	29	97
	1:750	20	12	60
Normal P-1	1:10	30	7	23
	1:100	25	5	20
	1:200†	26	3	12
	1:500	25	2	8
Leukemic P-1	1:10	9	9	100
	1:100	10	10	100
	1:200†	25	24	96
	1:500	24	10	42
Leukemic P-2	1:10	10	10	100
	1:100	10	10	100
	1:200	9	8	89
	1:500	10	0	0
Normal nuclei	1:10‡	34	4	12
	1:50	20	0	0
Leukemic nuclei	1:10‡	34	26	76
	1:50	20	1	5

Serum dilutions used for comparisons are indicated in Table II.

son of the nuclei antisera demonstrated the markedly enhanced cytotoxic effect of the leukemic.

It may be pointed out here that the "infectious property" of the leukemic cells is contained within the cell. A standardized cell suspension was divided and one part stored in the refrigerator, while the other half was washed 3 times with phosphate buffered-physiological saline solution, and the cells suspended in buffered solution equal to the original volume of physiological saline. One group of mice received the original suspension; the other, the same dose of washed cells. There were no survivors in either group and the time of death was almost identical. No blood or tissue constituent therefore seems necessary for the *in vivo* establishment of the leukemic

TABLE III.
Showing Results of Challenge Dose of 100,000 Leukemic Cells Given to Groups of Mice Surviving Inoculation of Cell-Serum Mixtures.

Mice surviving after cells + antiserum	No. of mice	No. of survivals	% of survivals
Normal mitochondria*	5	0	0
,, P-1	10	0	0
,, nuclei	10	0	0
Leukemic mitochondria†	5	0	0
,, P-1	10	2	20
,, P-2	10	2	20
,, nuclei	10	2	20

* Ten other mice received 10,000 cells. None survived.
† ,, ,, ,, ,, ,, ,, ,, ,,

cell. Furthermore this observation substantiates the use of the term "cytotoxic" in reference to the action of the antiserum. A further observation concerns the question of immunity in mice surviving the antiserum-cell mixture for 45 days. Groups of such mice were given challenge doses of 100,000 leukemic cells. The results of these second inoculations are given in Table III. While the individual groups are small it is apparent that there is a significant difference between the survival rates of the animals "immunized" with leukemic cells exposed to normal antisera, and leukemic cells incubated with leukemic antisera. None of the mice in the first group of 25 survived, while 6 of the 34 making up the second group remained well for the 45 day period.

By use of the point binomial it can be shown that the probability of 6 survivals out of 34 animals is less than 1 in 100,000 if the true death rate is accepted to be 99% (Table I.). Other mice which had also survived the first 45 day period were sacrificed and their spleens examined. Based on macroscopic appearance and actual weight, the spleens would not be classed as leukemic. Pooled sera of other survivors did not protect mice in experiments employing the standard dose of 100,000 cells.

Summary. Antisera to both normal and leukemic spleen cell components exhibit cytotoxic effects for leukemic cells, upon *in vitro* exposure. In every instance the leukemic antisera were much more effective than the corresponding normal antisera. These differences were greater than those obtained by complement fixation procedures. The anti-mitochondria sera exhibited the highest "titers" but the least difference between the normal and leukemic. The most significant differences occurred between the leukemic and normal P_1 antisera, with the leukemic antiserum showing 8 times the activity of the normal. Highly significant differences were also demonstrated between the nuclei antisera. The leukemic antiserum proved over 6 times as effective as the normal. A slight active immunity was demonstrated in mice surviving the combination of leukemic cells and leukemic antisera.

Received October 7, 1949. P.S.E.B.M., 1949, v72.

Experimental Dental Caries. XIV. Further Studies on Effect of Certain Quinones.* (17537)

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The investigations on the inhibitory action of certain quinones, with and without vitamin K activity, on acid formation *in vitro*, as well as the contradictory findings on the effect of vitamin K in the prevention of dental caries have been recently reviewed by Dam.(1) In order to study further this problem we(2) carried out an experiment in which was studied the effect of 3 quinones with, and 3 without vitamin K activity, on dental caries activity in hamsters. The results showed that none of the compounds tested induced any significant decrease of dental caries. However, in the discussion of the results obtained we indicated the advisability of testing in a future experiment the effect on caries activity of various quinones and related compounds having different ability to inhibit acid production *in vitro*, and using larger amounts of the compounds than those previously used. It was stated that although in case of obtaining favorable results the implications of such an experiment would not favor the use of quinones, due to their toxicity, in the prevention of dental caries, a study of this kind should indicate how far the ability of certain quinones to inhibit acid production *in vitro* would be paralleled by a corresponding ability of these compounds to decrease caries activity. Therefore the findings thus obtained should contribute to a proper evaluation of the actual relationship between acid production and the development of caries. We are here reporting the results of such an experiment, which was carried out after the inhibitory power of certain quinones on acid production *in vitro* had been determined.

Experimental. First, an *in vitro* experiment was carried out in order to determine the inhibitory power of a series of quinones and related compounds, with and without vitamin K activity, on acid formation by certain oral bacteria. As a source of acidogenic bacteria was used pure cultures of 2 strains of lactobacilli (L_1 and L_2) and streptococci (S_1 and S_2) isolated from the deepest portion of a human carious lesion. The substrate used for the growth of streptococci consisted of casein-peptone broth plus 2% glucose. For the lactobacilli was used the same medium supplemented with yeast autolysate. The effect of 13 quinones and related compounds, 6 with and 7 without vitamin K activity, on the growth of the isolated bacteria was tested. 2-methyl-1,4-naphthoquinone (menadione) was used in the amount of 2 mg per 100 cc of substrate; the other substances were tested in the same concentration calculated on a molecular basis. In order to avoid the autoclaving of the compounds to be tested, they were added to the sterilized substrate in the tubes, dissolved either in sterile water or in an easily evaporable organic solvent such as acetone. The tubes were inoculated and then all were incubated with occasional shaking at 37°C for 18 to 20 hours, at which times the pH of the tubes, including the non-inoculated and inoculated controls, was determined with a glass-electrode potentiometer. The results obtained are presented in Table I.

It can be seen from this table that of the quinones derivatives with vitamin K activity methylnaphthoquinone, methylnaphthohydroquinone and methylnaphthohydroquinone disuccinate markedly inhibited acid formation by lactobacilli, and afforded complete inhibition of acid production by streptococci. On the other hand, the tetrasodium and dicalcium salts of methylnaphthohydroquinone diphosphate, as well as the disodium salt of methylnaphthohydroquinone disulfate, all of which have the same vitamin K activity as the first 3 substances, did not inhibit acid production by any of the bacteria tested. This marked inhibitory power of methylnaph-

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* This work was supported by a grant from Rask-Ørsted Fondet.

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TABLE I.
Comparison of Inhibition by the Compounds Tested of Acid Formation by Lactobacilli and Streptococci.

Kind of acidogenic bacterium		Lactobacillus L_1	Lactobacillus L_1	Lactobacillus L_2	Streptococcus S_1	Streptococcus S_1	Streptococcus S_2
No. of bacteria inoculated per tube (10 cc)		1,200	200,000	400	4,400	20,000	10,000
Incubation time (hr)		20	18	20	20	18	18
pH of non-inoculated tubes		6.95	6.86	6.95	6.89	6.89	6.89
Compounds added to inoculated tubes	Vit. K activity	pH of inoculated and incubated tubes					
Control		4.70	4.4	4.5	4.2	4.3	4.3
2-methyl-1,4-naphthoquinone (menadione)	+		6.3	6.4	6.9		
2-methyl-1,4-naphthohydroquinone	+		5.7		6.9		
2-methyl-1,4-naphthohydroquinone diphosphate tetrasodium salt (32.02% H_2O)	+		4.6		4.2	4.3	4.3
2-methyl-1,4-naphthohydroquinone diphosphate dicalcium salt (9.5% H_2O)	+	4.72	4.6	4.5	4.2	4.3	4.3
2-methyl-1,4 naphthohydroquinone disulfate disodium salt, $2H_2O$	+		4.6		4.2		
2-methyl-1,4-naphthohydroquinone disuccinate	+		5.6	6.4	6.9		
Benzoquinone	—	4.75	4.5	4.7	4.2	4.4	4.3
Hydroquinone	—	4.80	4.8	4.8	4.2	4.4	4.3
Quinhydrone	—	5.41		4.5		4.5	4.3
1,2-naphthoquinone	—	4.69		4.3		4.6	4.4
2-hydroxy-1,4-naphthoquinone	—	4.50		4.1		5.3	5.1
2,3-dichloro-1,4-naphthoquinone	—	5.29	4.5	6.4	4.3	6.0	4.8
Anthraquinone-2-sulphonic acid	—	4.62	4.7	4.5	4.2	4.4	4.3

TABLE II.
Compounds Added and Caries Activity in the 5 Groups.

	Group 1 5 +4	Group 2 5 +5	Group 3 5 +5	Group 4 5 +4	Group 5 5 +5
Kind and Amt. of each substance added to 100 g of cariogenic diet	Control	280 mg 2-methyl-1,4-naphthohydroquinone diphosphate dicalcium salt (9.5% H_2O)	223 mg 2-methyl-1,4-naphthohydroquinone disuccinate	168 mg anthraquinone-2-sulphonic acid	148 mg 2-3-dichloro-1,4-naphthoquinone
Vit. K. activity		+	+	—	—
Inhibits acid production *in vitro*		—	+	—	+
Av. No. of molars affected	9.6	9.6	9.2	8.4	8.3
Stand. dev.	±0.6	0.3	0.4	0.6	0.1
Av. No. of carious lesions	15.9	15.4	15.5	12.6	13.8
Stand. dev.	±2.0	1.0	2.0	1.6	1.1
Avg caries scores	8.6	8.7	13.4	15.2	7.8
Stand. dev.	±1.1	0.8	4.3	6.2	0.2

thoquinone, methylnaphthohydroquinone and methylnaphthohydroquinone disuccinate on acid production agree with the marked antibacterial effects *in vitro* of the same compounds reported by other investigators.(3,4) Of the 7 compounds with none or negligible vitamin K activity, only dichloronaphthoquinone showed in some cases a considerable inhibitory power on acid production, and hydroxynaphthoquinone inhibited slightly acid formation by streptococci.

Considering the results of these *in vitro* studies, in order to compare the effects of quinone derivatives, with and without vitamin K activity, having marked or none inhibitory action on acid formation), 3. anthraquinone-the following compounds were used in the caries experiments: 1. dicalcium salt of 2-methyl-1,4-naphthohydroquinone diphosphate (with vitamin K activity and without inhibitory action on acid production), 2. 2-methyl-1,4-naphthohydroquinone disuccinate (with vitamin K activity and with marked inhibitory action on acid formation), 3. anthraquinone-2-sulphonic acid without vitamin K activity and without inhibitory action on acid formation, and 4. 2-3-dichloro-1,4-naphthoquinone (without vitamin K activity and with inhibitory action on acid formation).

Fifty newly weaned hamsters from an inbred colony were littermate distributed into 5 groups (5 males and 5 females in each), and were reared in screen bottom cages without bedding for 100 days on the following basal diet to which was added in each case the quinone derivative indicated in Table II: Salt mixture† 1%, alfalfa meal 2%, Brewer's yeast 5%, powdered whole milk 22%, finely ground yellow corn 25%, and finely powdered sucrose 45%. All the quinone derivatives were supplied in equal molecular concentrations. The groups were given water *ad libitum*, and were weighed weekly. On completion of the 100-day experimental period the animals were sacrificed and autopsied. The molars were prepared for examination and the carious lesions were recorded and scored as previously.(5)

Results. At the levels given most of the

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3. Armstrong, W. D., Spink, W. W., and Kahnke, J., PROC. SOC. EXP. BIOL. AND MED., 1943, v53, 230.

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† The salt mixture used was McCollum's Salt Mixture No. 185, supplemented with 13.5 mg KI, 139 mg $CuSO_4$, $5H_2O$, and 556 mg $MnSO_4$, $4H_2O$ per 100 g.

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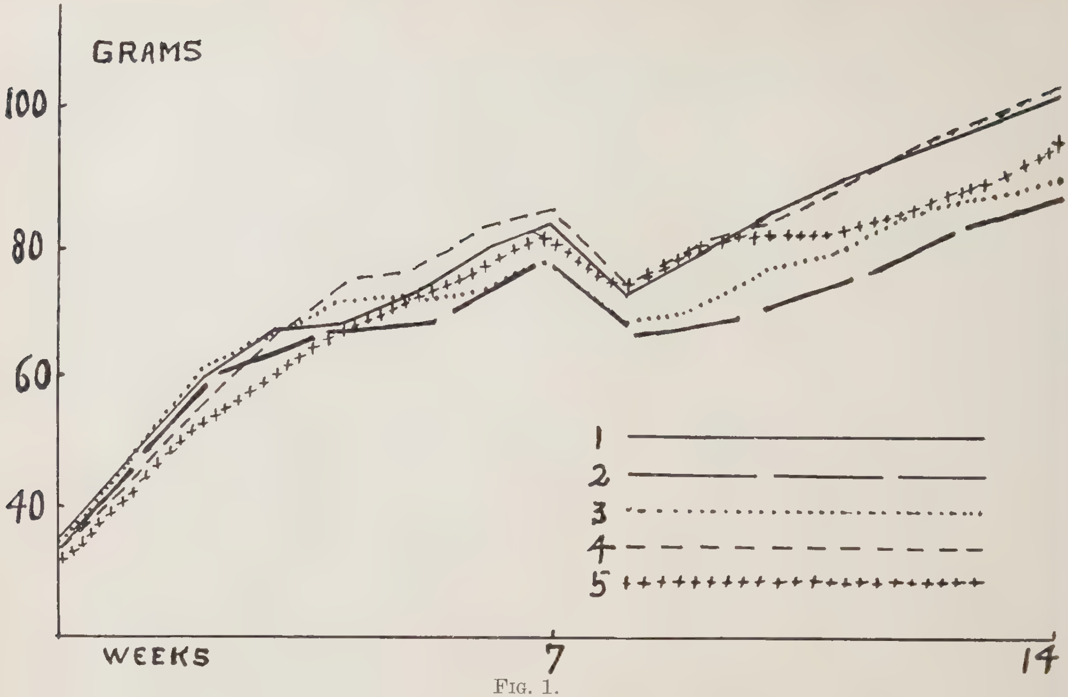

FIG. 1.

Average growth curves of the 5 groups. Group 1, control. Group 2, dicalcium salt of 2-methyl-1,4-naphthohydroquinone diphosphate. Group 3, 2-methyl-1,4-naphthohydroquinone disuccinate. Group 4, anthraquinone-2-sulphonic acid. Group 5, 2-3-dichloro-1,4-naphthoquinone.

quinones derivatives used showed to be toxic through an inhibition of growth. Fig. 1 shows that in increasing order dichloronaphthoquinone (group 5), methylnaphthohydroquinone disuccinate (group 3), and dicalcium salt of methylnaphthohydroquinone diphosphate (group 2) were toxic. On the other hand, anthraquinone sulphonic acid (group 4) did not induce any inhibition of growth as compared with the control (group 1). Table II shows, besides the compounds used, their vitamin K activity and their ability to inhibit acid production, the caries activity of the 5 groups. Beneath the average number of carious molars, carious lesions, and caries scores are presented the standard deviations of the means. The results presented in this table show that none of the quinone derivatives tested decreased the incidence or extent of caries. On the other hand, the higher average caries scores exhibited by groups 3 and 4 are not significant since these increased caries scores were due only to the exceptionally high caries activity of one animal in each of these groups. This is clearly seen from the much higher standard deviations of the caries scores in these two groups.

Discussion. This experiment confirms the negative findings of our previous study on this subject:(2) dicalcium salt of methylnaphthohydroquinone diphosphate and dichloronaphthoquinone were used in both experiments, but in none of them these compounds were able to decrease caries activity, in spite of the fact that in the present study the substances were used in amounts four times higher than those used in the first experiment. Furthermore, although most of the quinone derivatives used in the previous(2) and present studies have shown to inhibit considerably acid formation(6-10) and to have antibac-

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7. Armstrong, W. D., and Knutson, J. W., PROC. SOC. EXP. BIOL. AND MED., 1943, v52, 307.

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terial effects(3-4) *in vitro*, none of them decreased caries activity. Likewise, except dichloronaphthoquinone, all the other substances used in both experiments are readily soluble in water, while methylnaphthoquinone is very slightly soluble. Moreover, of the compounds tested in the present experiment methylnaphthohydroquinone disuccinate deserves special mentioning; it is easily soluble in water, inhibits markedly acid production, and has a definite antibacterial effect.(4) However, in spite of these properties it did not decrease at all the incidence or extent of carious lesions.

Assuming that the quinones tested should have had *in vivo* the same action as they showed *in vitro*, the results of the present studies might be interpreted in various ways. 1. Presuming that the initial lesion in dental caries is primarily due to disintegration of the mineral part of the enamel by acids produced by bacteria in the dental plaque, it could be said that the quinone derivatives tested did not decrease caries activity because they were unable to penetrate the dental plaque, and there exert their inhibitory action on acid formation. However, there have been made only a few studies on the degree of, and factors influencing the penetration of acid-formation inhibiting compounds into the dental plaque *in vivo*. Muntz and Miller(11) have studied the inhibition by certain compounds of the bacterial metabolism of intact dental plaques and of homogenized plaque material, as well as the degree of permeability of the plaques to various substances. They found that the metabolic activity of the intact plaque is inhibited more slowly than that of an equivalent quantity of homogenized plaque material, and that certain very diffusible substances such as urea and glucose penetrated intact plaques slowly. On the other hand, in a previous study on the effect of dietary lactic acid on dental caries activity (given in the food or in the water) we(12) found clear signs of acid action, combined or not with caries, at the very bottom of the occlusal fossae. This showed that the dietary lactic acid at the concentration given had actually penetrated the intact dental plaques. These facts indicate the complexity of the problem and the need for more fundamental research on the ability to penetrate the intact dental plaque of various substances known to influence the development and/or progress of caries.

2. Another factor which should be considered is that the acid-formation inhibiting power of the quinone derivatives used may have been annulled by the action of some other substances, either of dietary or systemic origin. A 3rd possibility is that the quinone compounds at the levels given, both in the present and in the preceding experiments,(2) may not have encountered in the oral environment sufficient water available for their dissolution and subsequent action. A 4th possibility is that the quinone derivatives which inhibited acid production *in vitro* may have also, at the concentrations given, inhibited acid production in the mouth, inside and outside the dental plaque. In such a case, since there was no decrease of caries activity in any of the groups which were given quinone compounds, this would mean that acid formation should not play any primary role in the development of dental caries since its inhibition would not decrease the incidence of the disease. However, none of the foregoing considerations goes beyond the field of speculation since most of the fundamental processes related to dental caries which take place in the organism, inside and outside the oral cavity, have not yet been adequately and sufficiently studied, and therefore they are not as yet well understood.

Thus, the various studies(6-10) *in vitro* carried out so far, using saliva-glucose mixtures, have demonstrated beyond doubt the acid-formation inhibiting action of a good number of quinone derivatives, with and without vitamin K activity. The first part of the present studies has shown once more such an inhibitory power of certain quinone compounds. Furthermore, the antibiotic effects of various quinone derivatives on a good number of bacteria permanently found in the

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12. Granados, H., Glavind, J., and Dam, H., *J. D. Res.*, 1949, v28, 282.

oral cavity have also been demonstrated.(3,4,13,14) On the other hand, the few clinical studies on the effect of 2-methyl-1,4-naphthaquinone incorporated into chewing gum, on caries activity have been contradictory since the decrease in incidence of new carious lesions reported by Burrill and coworkers(15) could not be substantiated by the U. S. Professional Service Schools.(16) Moreover, the previous experimental investigations carried out in rats(17) and hamsters,(2) as well as the present studies, on the effect of quinone derivatives with and without vitamin K activity, in the control of dental caries have all been negative. Therefore, an impartial analysis of the results obtained in clinical and experimental studies on this subject, demonstrate that vitamin K active compounds or other quinone derivatives do not show any promise of controlling caries, and therefore the use of these compounds as a control measure against human dental caries should be discouraged. Certain previous suggestions on the possible beneficial effect of vitamin K in the control of caries(18) were the product of theoretical considerations which have not been substantiated by actual experiments.

Summary. Further studies on the effect of different quinone derivatives with varying degrees of acid-formation inhibiting power, as determined *in vitro,* and with or without vitamin K activity, on dental caries activity were carried out in hamsters. In these studies the following quinone derivatives were tested: Dicalcium salt of methylnaphthohydroquinone diphosphate, methylnaphthohydroquinone disuccinate, anthraquinone sulphonic acid, and dichloronaphthoquinone. The compounds were used in higher amounts than those used in the previous experiment.

The results showed that none of the quinone derivatives tested exerted beneficial effect against dental caries activity, as compared with the control group. Thus the present experiment confirms the negative findings of previous studies, clinical as well as experimental, on this subject, and indicates that the use of vitamin K active compounds or other quinone derivatives as a control measure against human dental caries should be discouraged. Furthermore, some other implications of these studies have been discussed.

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Received November 11, 1949. P.S.E.B.M., 1949, v72.

The Treatment of *Trichomonas vaginalis* Vaginitis with Aureomycin.
(17538)

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Since we(1) had been successful in treating amebiasis with aureomycin it was thought possible that this antibiotic might be useful in other parasitic infestations. It was recalled that many amebicidal preparations are effective against *T. vaginalis.* Subsequent *in vitro* studies revealed that aureomycin is likewise trichomonicidal. Therefore, it was decided to investigate the action of the local application of aureomycin into the lower female genital tract in *T. vaginalis* vaginitis. A powder for vaginal insufflation was prepared by adding

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aureomycin hydrochloride to powdered talc (U.S.P. Merck). The first 6 patients were treated with talc alone. In each of these cases the vagina was insufflated with 2 g of powdered talc on 2 consecutive days and again on the fourth day. At the end of this period the vaginal secretions were negative for *T. vaginalis* in all 6 cases. Three days following cessation of therapy, the vagina contained *T. vaginalis* in 5 of the 6 cases. In the sixth patient vaginal secretions did not become positive until the eighth day. The leukorrhea and vaginal inflammation had persisted.

Aureomycin in dosages of 2 grams a day for 7 days by mouth has been ineffective in the treatment of this condition. Through the cooperation of the Medical, Gynecological, and Obstetrical Services of the John Gaston Hospital 54 case of *T. vaginalis* vaginitis were obtained for treatment. Twelve of these patients were pregnant and were referred for treatment by the Obstetrical Service. The diagnosis in each case was established by the demonstration of the organism microscopically in the wet-mount preparation.

The preparation for each vaginal insufflation consisted of 500 mg of aureomycin and 2 g of powdered talc. A Holmes insufflator equipped with a rubber cuff and detachable end-piece was employed. The preparation was sprayed evenly over the cervix, vagina, introitus and vulva. Following each treatment patients were instructed to wear a vulvar pad until the next treatment and to refrain from douches or intercourse.

Thirty-one symptomatic non-pregnant patients and 11 asymptomatic non-pregnant patients were insufflated as described above on the first, second, fourth, and sixth days of therapy. Following this the patient was instructed to insert one 250 mg gelatin capsule of aureomycin every other night deep into the vagina for 2 weeks. Twelve symptomatic pregnant patients were similarly insufflated on the first, second, third, fourth, sixth, and eighth days of treatment. Subsequently a 250 mg capsule of aureomycin was inserted each night for 2 weeks. Of the 31 symptomatic, non-pregnant cases there was objective evidence of relapse or reinfection but no return of symptoms in 3 cases. These 3 patients were subsequently retreated successfully. There were no recurrences among the 11 asymptomatic non-pregnant women treated. The rapid subjective and objective improvement of the pregnant cases was most gratifying. These patients were treated more vigorously than the others since it is well established that the disease is much more stubborn to any form of therapy during pregnancy. Three of the 12 pregnant cases showed evidence of reinfection or relapse following therapy, but in only one was there a return of symptoms. This last patient was extremely resistant to treatment and responded only after 3 courses of therapy. All of the 54 patients have been followed at biweekly intervals for 2 to 3 months since stopping treatment.

No significant toxic reactions to intravaginal aureomycin were noted. In 9 of 54 cases mild discomfort, as pruritus, burning or mild intravaginal pain was experienced. This usually occurred after the first or second application of aureomycin and could not always be definitely attributed to the drug. There was no demonstrable adverse effect on pregnancy. It is noteworthy that no objective evidence of local tissue reaction to aureomycin could be demonstrated on physical examination. Moreover, there is no need for concern over any cumulative effects of aureomycin as contrasted to the arsenicals since the small quantities of the drug absorbed from the vagina are rapidly excreted in the urine and bile. Blood levels of aureomycin (done in only 2 cases) 3 hours after vaginal insufflation of 500 mg in talc were in the neighborhood of 0.12 μg per cc of blood. Twelve of the cases in this series have been followed by approximately 6 months. In only 2 have trichomonads reappeared.

Received December 15, 1949. P.S.E.B.M., 1949, v72.

Effect of Purified Growth Hormone on Tumor Growth.* (17539)

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Alterations of hormonal processes have been suspected as being involved etiologically in neoplastic diseases. Several early experiments(1,2) indicate that anterior pituitary extracts stimulated the growth of inoculated tumors, while conflicting findings exist regarding the therapeutic success of similar extracts in inoperable cancer patients.(3,4) Stern(5) believes that "the anterior pituitary secretes two groups of hormones which are antagonistic as to their effect on tumor growth." Extracts containing the growth hormone principle are thought to stimulate neoplastic growth(6,7) while gonadotropic factors seem to inhibit tumor growth.(8,9) These divergent views may be attributed to differences in the experimental tumor and species of animal employed, as well as to the variable nature of the endocrine preparation used. This study was prompted by the recent availability of purified growth hormone (Armour[†]) in response to a new method of preparation,(10) and was undertaken to determine whether tumor growth could be influenced by the hormone. The activation of normal growth under the influence of the anterior hypophyseal hormone is well-known.(11,12)

Inbred "A" strain mice of both sexes were used. The animals weighed between 18 and 24 g, and were 2 to 3 months old. The mice received a diet of commercial pellets (Purina Lab Chow), weekly supplements of fresh lettuce, and were permitted access to food and water at all times. Tumors were transplanted by the trocar method, each animal receiving a fragment of neoplastic tissue in the right inguinal region. Sarcoma 37 and the familiar mammary adenocarcinoma were the test tumors. The latter tissue was not as satisfactory as the sarcoma, since there was the occurrence of late visceral metastases,(13) a tendency towards the formation of cysts containing white stringy material, and an earlier onset of visible necrosis than in the transplantable sarcoma.

Six days after tumor transplantation, the hormone therapy began. The animals included for the experiment had tumors of the following dimensions, 3.5 ± 1.5 mm × 3.0 ± 1.0 mm × 2.5 ± 0.5 mm. For the first two days, the daily dose was 0.1 mg growth hormone[‡] in 0.2 ml distilled water (pH adjusted to 7.4 with sodium bicarbonate); from the third to the tenth day, the daily dose was 0.2 mg; the dose was increased to 0.5 mg on the eleventh day and continued at this level for 4 more days at which time the experiment was terminated. Throughout the experiment, the controls received corresponding amounts of crystalline bovine plasma albumin

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[*] Aided by a grant from the National Cancer Institute, U. S. Public Health Service.

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[‡] A preliminary assay on plateaued female Long-Evans rats (about 200 g) showed that as little as 0.5 mg of the hormone caused a net increase of 10 g in weight over the controls in a 24-hour period.

TABLE I.
Effect of Purified Anterior Pituitary Growth Hormone on Tumor Growth.
Wt (in g) at autopsy.

Sarcoma 37						Mammary adenocarcinoma					
Controls			Hormone-treated			Controls			Hormone-treated		
3.45	0.56	1.68	2.14	2.29	0.91	2.37	2.55	2.95	1.60	2.30	1.90
3.02	0.22	1.88	2.25	1.69	1.29	2.03	1.55	1.75	1.10	1.15	1.70
2.27	0.55	1.76	2.98	2.90	1.32	1.30	1.30	1.53	2.40	1.10	0.70
1.89	1.07	2.42	2.42	2.13	2.01	1.60	1.35	2.42	1.00	1.28	0.95
1.71	2.58	1.58	1.41	0.29	1.37	1.20	0.45	1.35	1.75	0.47	1.25
1.64	3.57	1.36	1.37	0.41	2.01	1.55	1.40	1.58	1.95	1.43	0.65
2.14	2.67	1.23	2.00	2.83	2.17	0.80	1.10	1.57	1.20	0.63	1.20
1.06	2.14	1.15	1.74	0.76	1.74	0.77	0.70	0.90	1.25	0.72	1.10
0.73	1.92	1.08	2.01	1.48	0.94	0.13	0.60	1.28	1.25	0.49	0.55
0.88	2.02	1.69	1.39	1.12	1.70	0.12	1.40	0.87	0.75	1.03	0.70
1.73 ± 0.15*			1.70 ± 0.13			1.35 ± 0.12			1.19 ± 0.095		

* Mean values ± standard error.

(Armour). The mice receiving growth hormone showed a marked increase in weight during the first week of therapy. After this time, when the tumors were becoming necrotic, all groups suffered weight losses.

The animals were killed by decapitation; the tumor was peeled away from the subcutaneous connective tissue of the host, the interior exposed, and all visible necrotic material removed. The weights of the tumors are recorded in Table I.

It can be seen that the purified growth hormone did not show any appreciable effect, stimulatory or inhibitory, on the transplantable tumors used. Histological studies, which were kindly performed by Dr. E. Lowenhaupt of the Department of Pathology, revealed that tumors of all groups of animals, hormone-treated and controls, were of essentially similar appearance as regards relative proportions of necrotic tissue, numbers of mitotic figures and cell appearance.

Conclusion. Purified growth hormone does not influence the growth of sarcoma 37 and the mammary adenocarcinoma in "A" strain mice.

Received November 29, 1949. P.S.E.B.M., 1949, v72.

Activation of Purified Prothrombin.* (17540)

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The activation of prothrombin has probably been discussed more extensively than any other problem in blood coagulation. It is an important process and exceedingly complex in nature. Calcium, thromboplastin, Ac-globulin and platelet derivatives participate in this vital function; and although it is not exactly clear how the activators work, it is apparent that they may be divided into two main classes; namely, those with thromboplastin activity in the traditional sense and those with accelerator or thromboplastin co-factor activity. Either class alone is a poor activator of purified prothrombin, but together they cause rapid conversion. To understand more exactly what forces these substances bring to bear on prothrombin it is essential that they be obtained in purified form and in sufficient quantity for study. This still needs to be accomplished, but in the meantime it may be possible to obtain indirect information concerning the mechanism of their action. For example, this

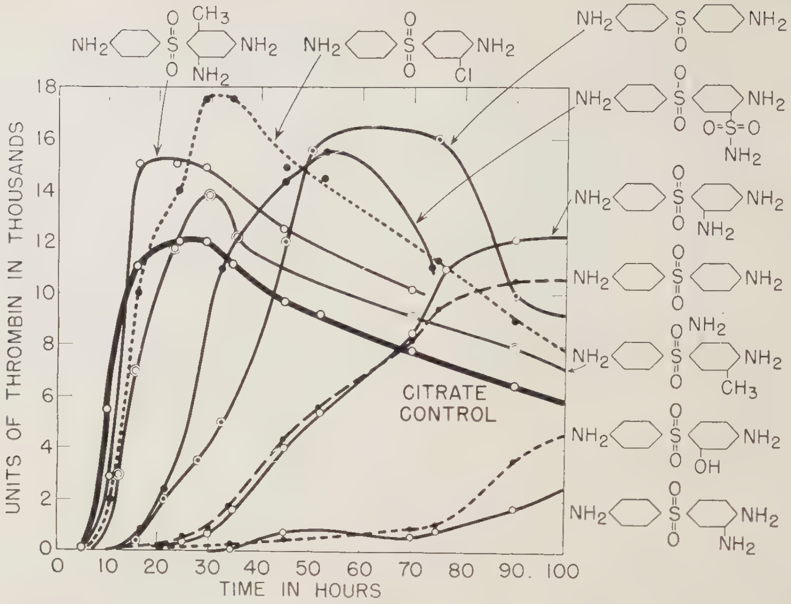

Fig. 1.

Activation of purified prothrombin in 25% sodium citrate solution, and of the same prothrombin in 25% sodium citrate solution together with a saturated solution of the organic compound indicated for each curve. The concentration of prothrombin was 18,000 units per cc.

paper describes prothrombin activation experiments in which sodium citrate, an organic compound of known structure, is the activating agent. This activation can be either blocked or synergized by certain diphenyl sulfones whose structure is also known. It should be possible to elucidate the nature of the forces required for prothrombin conversion by studying the action of these relatively simple compounds as activators and inhibitors, and thus by analogy formulate viewpoints concerning the more complex physiological activators. The work to be described is sufficiently promising to justify a hopeful outlook for the new attack on the problem of thrombin formation.

Experimental. Thrombin Analysis. Quantitative measurement of thrombin activity was done by the method of Seegers and Smith.(1)

Prothrombin. Purified prothrombin was obtained from bovine plasma by methods previously described.(2,3) Only high quality material was used. Generally the specific activity was 1200 units per milligram dry weight or more. It was free of Ac-globulin activity and contained no thrombin. On electrophoresis such products have a main boundary pattern comprising about 90% of the total. Quantitative determination of activity was by the 2-stage method.(4,5)

Diphenyl Sulfones. These were all synthesized in the laboratory and it is expected that the method of synthesis will be described in detail elsewhere. I wish to thank Leonard Doub for placing these compounds at my disposal. The crystalline compounds were added to the prothrombin dissolved in 25% sodium citrate solution. All are sparingly soluble and it required only a few milligrams to give a saturated solution with respect to the sulfone.

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No attempt was made to have the concentration of the sulfones equal on a molecular weight basis.

Prothrombin Activation. Powdered sodium citrate was added to an aqueous solution of prothrombin so that the concentration of sodium citrate was 25% and the concentration of prothrombin was about 1.5%. Then the crystalline sulfone was added in sufficient quantity so that crystals could be seen suspended in the prothrombin solution. The mixture was allowed to stand at room temperature, and samples were taken for thrombin analyses. The results presented in Fig. 1 were obtained with the same prothrombin preparation and, were obtained simultaneously so as to obtain the best possible comparable results with the various sulfones.

Results. With the 25% sodium citrate solution prothrombin activation was slow during the first 5 hours. After this induction period activation was rapid, and within a day the maximum yield of thrombin was obtained. This was, however, only 67% of the potential thrombin which could be obtained from the prothrombin with the use of calcium, thromboplastin and Ac-globulin. The thrombin titer was followed for 3 more days and showed a progressive decline similar to that commonly encountered with thrombin solutions which are not stabilized with glycerol or carbohydrates.(6) By adding 3, 4, 4′-triaminodiphenyl sulfone, or 2-hydroxy-4, 4′-diaminodiphenyl sulfone to the prothrombin in 25% sodium citrate solution the induction period was prolonged to more than a day and even thereafter only small amounts of thrombin formed so that in 5 days the thrombin yield was less than 25%. These compounds can thus be considered powerful inhibitors of prothrombin activation under the conditions of the experiment.

It is also possible to obtain the opposite effect; namely, an acceleration of prothrombin activation. The most interesting compound studied was 3-chloro-4, 4′-diaminodiphenyl sulfone. It prolonged the induction period about 2 hours. Then thrombin formation was far more rapid than with the citrate control and the final yield was almost 100% as compared with 67% for the control. The deterioration of the thrombin solution was at a rate typical of other thrombin solutions. Several other compounds were also found to be accelerators. Two of these, 4, 4′-diaminodiphenyl sulfoxide and 3-sulfonamide-4, 4′-diaminodiphenyl sulfone doubled the induction period but once thrombin began to form it continued until a high yield was obtained on the third day. Two others, 2, 4, 4′-triaminodiphenyl sulfone, and 4, 4′-diaminodiphenyl sulfone prolonged the induction period to one day. The thrombin titer then rose very slowly so that a 67% yield was obtained on about the fourth day.

Discussion. There are two practical benefits which can be derived from these experiments. (1) The preparation of thrombin from prothrombin is simplified. By adding 3-chloro-4, 4′-diaminodiphenyl sulfone to purified prothrombin in 25% sodium citrate the yield is increased to a maximum. There is thus no loss of an expensive prothrombin product, or remnants of proenzyme with the desired thrombin. (2) The preparation of prothrombin is simplified. In the purification procedures it has been difficult to eliminate thrombin from the final product. Failure to do so causes the prothrombin to be altered by the small amounts of thrombin.(7) This hazard can now be eliminated, for the ammonium sulfate used for fractionation work, like sodium citrate, causes slow activation of prothrombin, and this effect of ammonium sulfate can be blocked by adding small amounts of 3, 4, 4′-triaminodiphenyl sulfone to the ammonium sulfate solutions used in the fractionation procedures.

It has been discussed repeatedly that thromboplastin and Ac-globulin together activate prothrombin more rapidly and give a higher yield of thrombin than when the former is not supplemented with Ac-globulin. This general observation also applies to these artificial activators. Sodium citrate alone, like thromboplastin, acts slowly and the yield of thrombin is incomplete, but sodium citrate with 3-

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chloro-4, 4′-diaminodiphenyl sulfone, like thromboplastin with Ac-globulin, activates prothrombin more rapidly and the yield of thrombin is increased. The similarity between the action of the artificial activators and the physiological ones would seem to be of more than coincidental significance, even though the time intervals involve hours in one instance and minutes in the other.

Perhaps the most remarkable result of these experiments is the difference in action of 3, 4, 4′-triaminodiphenyl sulfone and 3-chloro-4, 4′-diaminodiphenyl sulfone. One has an amino group in the three position and the other a chlorine atom. One is an inhibitor and the other an accelerator. Thus, for one set of conditions, the difference between a "coagulant" and an "anticoagulant" has been reduced to a locus on the benzene ring where placement of an amino group produces one effect and a chlorine atom the opposite. This subtle detail is easily brought into focus, and perhaps it is here where we can hope to see, for the first time, what forces physiological anticoagulants and coagulants bring to bear on prothrombin.

Summary. Purified prothrombin can be activated by dissolving it in a 25 per cent solution of sodium citrate, but the yield of thrombin is usually low. By adding a small amount of 3-chloro-4, 4′-diaminodiphenyl sulfone to the activation mixture the yield of thrombin is increased to the maximum. Several other diphenyl sulfones also increase the yield. In contrast, 3, 4, 4′-triaminodiphenyl sulfone and 2-hydroxy-4, 4′-diaminodiphenyl sulfone act as inhibitors of the activation process.

Received November 9, 1949. P.S.E.B.M., 1949, v72.

Cultivation of Influenza Virus in the Chorio-Allantoic Membrane of Deembryonated Eggs.* (17541)

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Fertilized chicken eggs have proved to be an excellent medium for the growth of many viruses and other pathogenic agents. A number of viruses grow in the various tissues of the embryo and in the membranes enveloping it in an indiscriminate manner while others show specific affinities for certain structures in the egg. For a number of viruses, among them those of the influenza group, the chorio-allantoic membrane(1-3) is the main site of virus multiplication. Most other structures offer less favorable conditions. It seemed an obvious step, therefore, to use only the chorio-allantoic membrane *in situ* for virus growth and to eliminate the other constituents of the egg. For this purpose the following technic was found practicable.

Methods. Eggs which have been incubated for 14-15 days are candled and the outline of the air-sac is marked. One-half centimeter above this line the egg shell is cut with an electric drill and the piece of shell is removed. The free-lying shell membrane and the marginal zone of the shell both on the inside and outside along this opening are covered with hot paraffin. The shell membrane together with the piece of underlying chorio-allantoic membrane is then cut away with a pair of curved scissors. By gently tilting and rotating the egg the embryo and the yolk sac are poured out and their connections with the chorio-allantoic membrane are severed with

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* This investigation was conducted under the auspices of the Commission on Influenza, Armed Forces Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

[†] Aided by a fellowship from the Dazian Foundation for Medical Research.

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TABLE I.
50% Endpoints of Virus Titrations Done in:

Exp. No.	(a) 10-11-day eggs allantoically	(b) in 15-day "empty" eggs	(c) difference in logs
1	$10^{-7.46}$	$10^{-10.00}$	2.54
2	$10^{-7.57}$	$10^{-9.0}$	1.43
3	$10^{-7.33}$	$10^{-8.57}$	1.24
4	$10^{-7.66}$	$10^{-8.71}$	1.05
		Avg diff.	1.57

scissors. The chorio-allantoic membrane which is firmly attached to the shell membrane is the only structure remaining in the egg. The internal surface of the membrane is now thoroughly washed with 3 changes of cold 0.85% NaCl so as to remove any remaining yolk, albumen, and blood. After the egg is drained by placing it with the open end down on a sterile petri dish, it is filled with 10-40 cc of Tyrode's solution containing virus, and 10 units of penicillin and 40 μg of streptomycin per cc. The opening is closed with a sterile rubber cap, such as is used for centrifuge tubes, which is lined and then sealed to the shell with hot paraffin. The eggs in suitable trays are then put in a test tube roller which is kept in an incubator at 37° and makes six revolutions per hour. Fluid can be withdrawn from the eggs at desired intervals by piercing the rubber cap with a sterile needle attached to a syringe.

It has also been found possible to infect eggs first allantoically in the regular way and after the virus has been adsorbed to the membrane or as late as 48 hours after infection the contents of the egg are poured out. The interior is washed thoroughly and filled with Tyrode's solution. After further incubation at 37° virus increase can be demonstrated.

Results. Thus far, the PR8 strain of influenza virus has been used in the experiments. Hemagglutination titers of 1280-2560 in a pattern test were regularly obtained in the fluid after 24-hours' incubation when a 10-20 ml volume of 10^{-5} to 10^{-6} dilution of virus was used as inoculum. Under the same conditions a hemagglutination titer of 5120 is usually obtained with allantoic fluid of the complete egg infected with the PR8 strain. The specificity of the reaction is demonstrated by the inhibition obtained with specific serum against influenza virus, Type A. It is obvious, therefore, that the chorioallantoic membrane produces virus under the described conditions in much the same amounts as in the intact egg. Several tests were done in which the sensitivity of this method for detecting small amounts of virus was compared with that of allantoic inoculation. Ten-fold dilutions of allantoic fluid infected with PR8 strain were prepared in Tyrode's solution and inoculated in 0.1 ml amounts in 10-11 day old eggs allantoically and in 20 ml amounts in egg cavities prepared by the method described. Four eggs were used per virus dilution. The allantoic inoculations

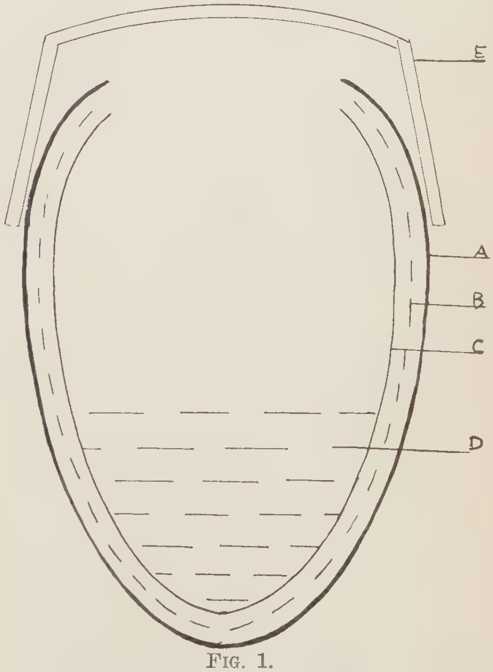

FIG. 1.
A. Egg shell. B. Shell membrane. C. Chorio-allantoic membrane. D. Tyrode's solution. E. Rubber cap.

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were always performed first. After 48 hours' incubation at 37°C all fluids were tested for hemagglutinins. Table I shows the results of several experiments in which the 50% end-points of infectivity obtained with the two methods were calculated by the method of Reed and Muench.(4) It is seen that the titers of allantoic fluid as measured by allantoic sac inoculation were always lower than those obtained with the prepared membrane alone. Since a much larger amount of fluid is introduced into the "empty" eggs, a correspondingly higher amount of virus is also inoculated. This may explain the greater sensitivity observed. Other explanations seem possible.

Discussion. The method may have various applications. It is possible to introduce larger amounts of fluid into the egg than by other methods and this may be of advantage when virus isolation from throat washings are undertaken. The influence of fluids of different composition on virus multiplication and the effect of inhibitors and accelerators of growth can easily be studied by this method which allows virus growth in a practically intact tissue. Moreover, the rate and character of growth of virus in the membrane may be more accurately studied. Such experiments are in progress. The method may have certain advantages for the production of vaccines as virus multiplication can be obtained in a medium of chosen composition, and the presence of urates in the virus fluid is excluded.

Summary. A method is described in which the chorioallantoic membrane of the egg *in situ* is used for the cultivation of influenza virus. The sensitivity of the method is demonstrated and various possibilities for its application are discussed.

Received November 29, 1949. P.S.E.B.M., 1949, v72.

Effect of Proteinuria on Toxicity of Mercurial Diuretics in the Rat. (17542)

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During the past 30 years, organic mercurial compounds have been used widely for the purpose of inducing diuresis. Because of their apparently low clinical toxicity, mercurial diuretics have been used with impunity in the presence of cardiac failure, and, even in the presence of impaired renal function, few clinicians have hesitated to administer them. Studies in animals(1-4) gave results which, while in some degree conflicting, nevertheless indicated that the agents could produce transient or permanent renal damage in doses which were comparable to those recommended for use in man. When clinical application of the mercurial diuretics was begun, it soon became evident that, although there was some indication of renal irritation,(4-6) the clinical toxicity was negligible,(7,8) at least in the absence of severe,

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* This study was aided by grants from the Division of Research Grants and Fellowships, National Heart Institute, United States Public Health Service, and from the Columbia Foundation, San Francisco. Meralluride sodium was provided by the Lakeside Laboratories, Milwaukee, Wis. Concentrated, salt-poor, human albumin was supplied by the American Red Cross.

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pre-existing renal damage. It was determined soon after their introduction that mercurial diuretics acted directly upon the kidney, (9-12) and there has been general agreement that diuresis results from depression of tubular function. The localization of this action in the tubule has been studied more recently, but has not been completely clarified. There are some indications that the effect is produced upon the proximal tubule,(13,14) whereas some evidence indicates an effect upon the distal tubule.(15) To further complicate matters, there is evidence for species differences in the effects of mercurial diuretics.(13,16)

Because it seemed probable that mercurial diuretics are potentially toxic substances which act by depressing tubular function, it seemed remarkable that such toxicity does not become clinically evident in patients with the nephrotic syndrome, when mercurial diuretics are used. Here, in a situation which is accompanied by tubular degeneration, it would seem reasonable to suppose that damaged tubule cells, seen as desquamated cells in the urinary sediment, would be more susceptible than normal cells to the toxic action of these compounds. Yet such patients often receive mercurial diuretics without ill effect, though often, in our clinical experience, without therapeutic effect. As a result of such observations and reflection, we became interested in studying the effect of proteinuria upon the toxic action of mercurial compounds.

TABLE I.
Effect of Proteinuria on Kidney Weight and Body Weight After Administration of Mercurial Diuretic.

Group No.	IP inject.	No. rats	Mercurial used	Mercurial inj. after IP inj. No.	Mercurial dose, mg Hg	No. rats surviving	Time kill interval survivors, days	Initial wt, g	Final wt, g	Kidney wt, mg	Anatomic diagnosis
1	Saline	6	Mersalyl	III	.26	6	1	153		1137	Nephrosis, acute, very slight
1a	Albumin	6	,,	III	.26	6	1	154		1226	Nephrosis, acute, very slight
2	Saline	12	,,	III	.79	12	1	158		1103	Nephrosis, necrotizing, acute, severe
2a	Albumin	12	,,	III	.79	12	1	159		1273	Nephrosis, acute, slight to moderate
3	Saline	14	Merallur.	III	1.95	10	7	153	134	1635	Nephrosis, acute, severe, advanced healing
3a	Albumin	13	,,	III	1.95	13	7	152	155	1183	Nephrosis, acute, slight
3b	,,	6	,,	II	1.95	6	7	154	154	1167	Nephrosis, acute, very slight
3c	,,	6	,,	I	1.95	1	7	150		2561*	Nephrosis, acute, severe, healing, slight hydronephrosis

* One surviving animal only.

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Methods and materials. This study was performed with 75 female rats, each weighing about 150 g. The organic mercurial diuretics studied were mersalyl-theophylline and meralluride sodium. Mersalyl-theophylline contains 39.6 mg Hg and 50 mg theophylline in 1 ml. Meralluride sodium contains 39.0 mg Hg and 48 mg theophylline in 1 ml. In the dosages tabulated (Table I) theophylline was present in the above ratio to the dose of Hg. The animals each received three 16 ml intraperitoneal injections, No. I at 9:30 A.M. and No. II at 4:30 P.M. on the first day, and No. III at 9:30 A.M. on the second day. For the control groups, the injection material was 0.85% sodium chloride solution. For the experimental animals, the injection material was 6% human albumin in 0.85% sodium chloride solution. Immediately after the designated intraperitoneal injection (Table I), the mercurial diuretic was administered intravenously, diluted to give the proper dose with 0.85% sodium chloride solution. The animals were then left undisturbed upon stock diet either until death, or for the interval specified, at which time they were killed by ether anesthetization and exsanguination from the abdominal aorta. The kidneys were removed promptly, except in the few animals that died during the night, decapsulated, sagittally sectioned, blotted, weighed, and dropped into 10% formalin. After fixing, paraffin sections were made and stained with hematoxylin and eosin for microscopic examination.

Results. The animals in Group 1 (saline + 0.26 mg Hg) and 1a (albumin + 0.26 mg Hg) appeared perfectly normal until killed. At autopsy they also appeared perfectly normal. There was no appreciable difference between the groups in kidney weight, except for the difference expected after albumin administration. The kidneys appeared normal in the gross, both externally and on sagittal section. In the microscopic sections there were minimal changes of acute nephrosis present in both groups. There was no convincing difference in the degree of nephrosis between the two groups. The animals in Group 2 (saline + 0.79 mg Hg) and 2a (albumin + 0.79 mg Hg) also appeared normal until killed. At autopsy, as in all the animals, there were no significant findings outside of the kidneys. In Group 2 the kidneys were somewhat smaller than normal, with slight pallor of the surface and the cortex. In Group 2a the kidneys were normal in size and the pallor was hardly evident, resembling the slight pallor that accompanies protein injections alone, in our previous experience. Microscopically, the kidneys in Group 2 showed definite and pronounced tubular damage (Fig. 1), while those in Group 2a showed slight damage of a much milder degree (Fig. 2).

In the animals of Group 3 (saline + 1.95 mg Hg), the findings were entirely different. These animals were obviously ill from the day of the mercurial injection and 4 of the 14

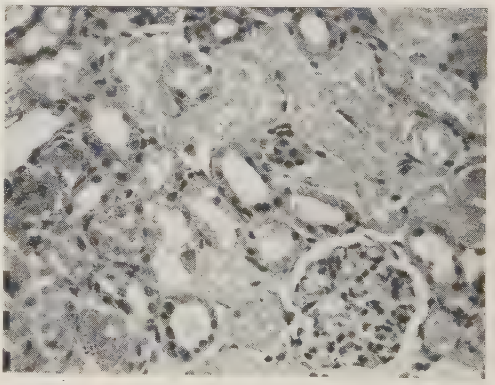

FIG. 1.
Section of kidney, Group 2: saline-injected animals, 1 day after mersalyl (0.79 mg Hg), showing acute necrotizing nephrosis. Hematoxylin-eosin stain, 240×.

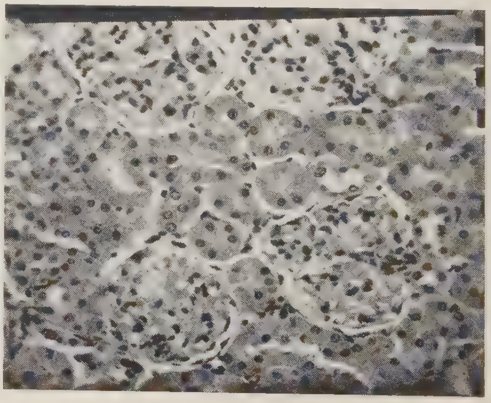

FIG. 2.
Section of kidney, Group 2a: albumin-injected animals, 1 day after mersalyl (0.79 mg Hg), relatively free of tubular damage. Hematoxylin-eosin stain, 240×.

animals died from the fourth to the sixth day after injection. At the end of a week, the survivors were severely ill, emaciated, and hyperirritable when disturbed. In this group the kidneys were greatly enlarged, with extremely pale granular surfaces. On sagittal section, the cortex was very pale and striated, while the medulla appeared to be relatively well preserved and of almost normal color. On microscopic examination, the tubular structure was disrupted, with appearance of severe toxic nephrosis in an advanced healing stage. (Fig. 3, 4). In sharp contrast, the animals in Group 3a (albumin + 1.95 mg Hg) appeared to be entirely normal until killed. All survived in good health and there was a slight gain in weight. At autopsy, the kidneys were normal in size and appearance except for the slight pallor mentioned under Group 2a. Microscopically there was a very slight degree of tubular damage (Fig. 5). In Group 3b (albumin + 1.95 mg Hg after inject. No. II) the animals were entirely comparable to those in Group 3a, and the kidneys were virtually indistinguishable (Fig. 6). The animals in Group 3c (albumin + 1.95 mg Hg after inject. No. I) were the most severely affected of all. Only one animal survived the

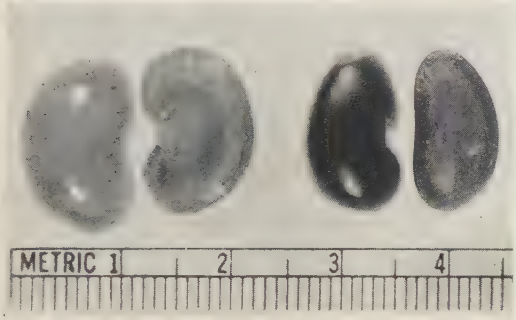

FIG. 3.
Kidneys, gross specimens. Left: Group 3, saline-injected animals, 7 days after meralluride sodium (1.95 mg Hg). Right: Group 3a, albumin-injected animals, same interval and same dose of meralluride sodium, injected after albumin injection No. III. Note difference in size and color of kidneys.

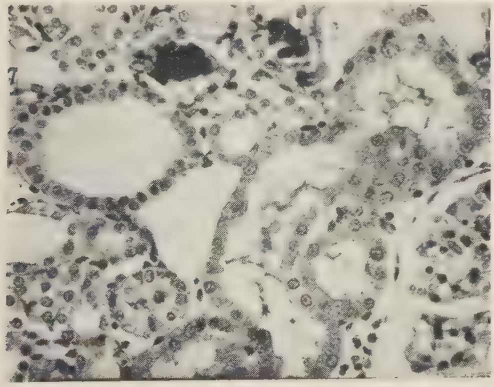

FIG. 4.
Section of kidney, Group 3: saline-injected animals, 7 days after meralluride sodium (1.95 mg Hg), showing necrotizing nephrosis with healing and deposits of calcific material. Hematoxylin-eosin stain, 240×.

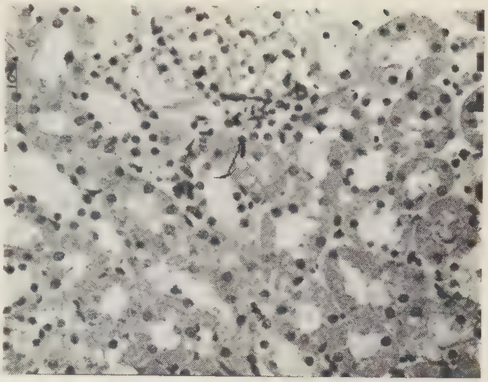

FIG. 5.
Section of kidney, Group 3a: albumin-injected animals, 7 days after meralluride sodium (1.95 mg Hg), administered at time of albumin injection No. III. Note relatively intact tubular structure with mild changes of acute nephrosis. Hematoxylin-eosin stain, 240×.

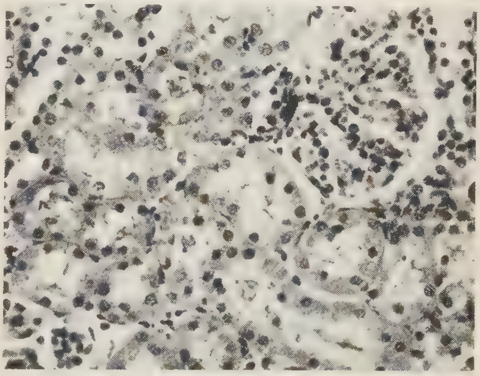

FIG. 6.
Section of kidney, Group 3b: albumin-injected animals, 7 days after meralluride sodium (1.95 mg Hg), administered at time of albumin injection No. II. Note that degree of damage is relatively slight, similar to that in Fig. 5. Hematoxylin-eosin stain, 240×.

full week, and it appeared moribund. The kidneys of those dying earlier showed pronounced enlargement, but those of the one surviving were most remarkably enlarged, almost chalky white, with a granular surface. On sagittal section, the entire kidney was similarly without color in both medulla and cortex. Microscopically, the tubular damage was profound and more severe than in any other group (Fig. 7).

Discussion. The data given here show conclusively that intraperitoneal administration of albumin to rats, in large doses, *prior* to the administration of a severely toxic dose of mercurial diuretic, effectively protects the kidneys from the toxic effects of mercury. On the contrary, when the protein is administered simultaneously with the mercurial, the toxic effect of mercury is enhanced. Many previous experiments with administration of protein, in the same dosage used in this study, have established the relation of proteinuria to the time of injections.(17,18) The first appearance of proteinuria after such injections has been interpreted as indicating that the maximum tubular capacity for reabsorbing protein has been exceeded, so that the rate of protein reabsorption is critically diminished. This coincides with the time of our injection No. II. Therefore, it seems probable that the diminished toxicity of mercurial diuretic at this time is associated with diminished tubular reabsorption of mercury as well as protein. On the other hand, it seems plausible to suppose that, during the interval from the first protein injection to the first appearance of proteinuria, the reabsorption of protein proceeds at a more rapid rate than normal, and that during this period the reabsorption of mercury is similarly increased, so that the toxicity of a given intravenous dose is enhanced. That mercurial diuretic is selectively concentrated in the kidney, in patients who have received mercurial diuretics(19) and in normal dogs,(20) is already known. Little is known about the precise molecular structure and physical properties of the mercurial diuretics. The prosthetic, mercury-containing portion of the complex is unstable except in combination with theophylline. However, the precise nature of the combination is not known, nor is the size of the complex known. We have tried to dialyse meralluride sodium, and found that after 48 hours virtually none had passed through a cellophane membrane. In an effort to ascertain whether the mercurial diuretics are bound by serum protein, meralluride sodium was incubated with 6% human albumin and then separation was attempted by precipitating the protein, using trichloracetic acid, phosphotungstic acid, and phosphomolybdic acid. In each case, the meralluride sodium did not appear to be bound by protein, all remaining in the supernatant fluid. Therefore, it would appear that inhibition of mercury reabsorption is probably not related to a union between the mercurial diuretic and protein, as such. Experiments are in progress to determine by cytological demonstration whether our hypothesis, that proteinuria reduces the quantity of mercury reabsorbed, is correct.

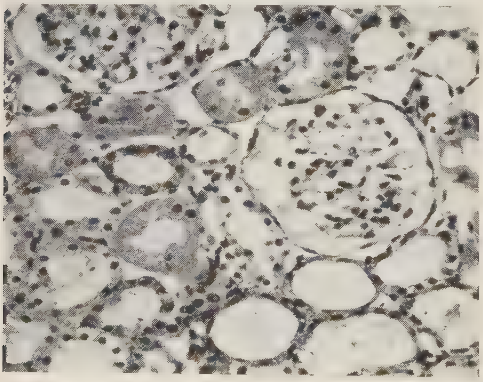

FIG. 7.
Section of kidney, Group 3c: albumin-injected animals, 7 days after meralluride sodium (1.95 mg Hg), administered at the time of albumin injection No. I. Note extreme severity of toxic nephrosis. Hematoxylin-eosin stain, 240×.

From these findings, it is suggested that our clinical experience concerning the unex-

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17. Addis, T., Final Report, Contract 338, Office of Scientific Research and Development, Committee on Medical Research, 1946.

18. Persike, E. C., and Ureen, H. J., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 339.

19. Forney, R. B., and Harger, R. N., *Fed. Proc.*, 1949, v8, 292.

20. Milnor, P., Burch, G., Ray, T., Threefoot, S., and Berenson, G., *Am. J. Med.*, 1949, v7, 412 (Proc.).

pected relative innocuousness and therapeutic ineffectiveness of mercurial diuretics in patients with renal disease may be related to the presence of marked proteinuria.

Addendum. After submission of this paper, we became aware of work done by Havill, Lichty, and Whipple,(21) showing that the tolerance of dogs for mercury bichloride was increased by frequent hemoglobin injections. It seems reasonable to assume that the mechanism of protection from the toxic action of the metallic ion was similar in this instance, since hemoglobin, like albumin, is reabsorbed by the proximal tubule cells until saturation of the reabsorptive capacity occurs, although Havill and his co-workers did not relate the protective effect to the occurrence of hemoglobinuria, which they did not measure, suggesting instead that the pigmented moiety of the hemoglobin molecule was responsible for the protection.

Summary. Administration of human albumin to rats, with resultant proteinuria, prior to administration of mercurial diuretics, sharply reduces renal toxicity of the mercurial. This effect is attributed to inhibition of mercurial reabsorption when the tubules are saturated with protein.

Simultaneous administration of human albumin and the mercurial enhances toxicity. This effect is attributed to the more rapid absorption of mercurial, concomitant with more rapid reabsorption of protein during the tubular loading phase.

The author gratefully acknowledges the invaluable technical assistance of Helen J. Ureen and Ruth Taniguchi. Dr. Lelland J. Rather reviewed the microscopic sections made for preliminary studies. The definitive studies were aided by Dr. Harry Goldblatt, who kindly reviewed and described the microscopic sections.

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21. Havill, W. H., Lichty, J. A., Jr., and Whipple, G. H., *J. Exp. Med.*, 1932, v55, 627.

Received November 14, 1949. P.S.E.B.M., 1949, v72.

Fetal Death and Maldevelopment Resulting from Maternal Vitamin A Deficiency in the Rat.* (17543)

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The course of pregnancy may be severely altered by feeding female rats a diet restricted in vitamin A, the severity of the alteration being roughly proportional to the degree of vitamin deprivation. Death and resorption of the fetuses, prolongation of pregnancy, and delivery of stillborn young were reported by Mason(1) and Cannon(2) who employed deficient diets with little or no supplement of vitamin A or carotene. Warkany and Schraffenberger(3,4) using similar diets supplemented with small amounts of carotene also noted a high incidence of fetal resorption; but, by interrupting pregnancy at various times from the 13th day to term, they were able to obtain a number of living and recently dead fetuses and newborns suitable for histologic study. The eyes of these offspring were found to bear several developmental anomalies, notably: folding and eversion of the retina, persistence of the choroidal fissure, absence of the ciliary body, and postlenticular fibroplasia. Thus, deficiency of vitamin A in the diet of female rats prior to and during pregnancy not only reduces fecundity by causing fetal death and resorption, but also causes developmental anomalies in many of the fetuses that escape intrauterine death.

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* Supported by a grant from the Nutrition Foundation, Inc., New York.

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2. Cannon, M. D., PROC. SOC. EXP. BIOL. AND MED., 1940, v44, 129.

3. Warkany, J., and Schraffenberger, E., PROC. SOC. EXP. BIOL. AND MED., 1944, v57, 49.

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The widespread interest aroused by the success of Warkany and his collaborators in producing congenital malformations by means of maternal vitamin A deficiency makes it desirable that the work be repeated in another laboratory. To date the only published account of attempts to repeat the experiment elsewhere was that of Jackson and Kinsey,(5) who did obtain a few newborn rats with ocular malformations; but the positive results of this investigation were of such limited nature as to cast doubt on the facility and regularity with which such defective young might be produced. One of us (J.G.W.) has been associated with Dr. Warkany in a morphologic and embryologic study of the malformed fetuses and newborns obtained from the experiments carried out in Cincinnati. Since certain material needed for further investigation of this subject (placentae and early embryonic stages prepared especially for histochemical study) was not readily available from Dr. Warkany's laboratory, it was decided to produce it in this laboratory. The present report relates the repetition of the original experiment and, in addition, presents some observations not previously recorded.

Experimental. Vitamin A deficiency was induced and maintained by the same diets and experimental procedures as were employed in Warkany's original experiments,(3,4) except for the quantity and method of administration of the carotene supplement, and a few other matters of incidental nature specified below. Rats of two strains were used, the Albino Farms and the Wistar; the latter had not been used previously for this type of experiment. Since no difference in reaction to the experimental procedures was detected in animals from the different sources, the matter of strain will receive no further mention. A total of 36 young females were raised from weaning on a semipurified, "preparatory" diet which contained very little vitamin A or carotene. To insure continued growth to maturity a 15 microgram dose of carotene in olive oil was administered orally each week. Presumably this dosage did not permit appreciable storage of vitamin A. When the animals had attained a body weight of about 160 g, each female was given an opportunity to breed with a male of the same strain fed an adequate diet. If mating occurred, as indicated by presence of sperm in the vaginal smear, the female was transferred to a more highly purified diet which was adequate in all known respects except for the lack of vitamin A. No further supplemental dosing with carotene was given. The day on the morning of which sperm were found in the vaginal smear was considered the first day of pregnancy. Eight control females were fed the same diets and subjected to the same procedures but were given weekly supplements of 150 μg of carotene, both prior to and during pregnancy.

From the experience of other investigators,(1,2,6) it was expected that a large percentage of the pregnant females on vitamin A deficient diets would resorb all of their conceptuses at some time before term. On the other hand, Warkany and Roth(6) found that approximately 25% of the conceptuses which survived until the latter part of pregnancy were developmentally normal. The margin between fetal destruction and normal development, therefore, appears to be very narrow. One of the aims of the present experiment was to attempt to establish criteria by which one might predict ultimate destruction of the conceptuses, thereby making it possible to obtain prior to death embryos which were destined to undergo resorption later. Mason(1) observed that the presence of cornified cells in the vaginal smear during pregnancy was frequently associated with subsequent fetal death and resorption. Warkany and Schraffenberger(4) considered excessive amounts of blood in the vaginal smear and a loss of weight by the mother as indications that resorption had already begun. It was hoped that by carefully watching for all of these signs (cornified cells in the vaginal smear, excessive blood in the smear, and loss of weight

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5. Jackson, B., and Kinsey, V. E., *Am. J. Ophthal.*, 1946, v29, 1236.

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TABLE I.
Course and Termination of Pregnancy in Vitamin A Deficient and Control Rats.

Age of fetuses when removed or delivered, days	Days of pregnancy on which untoward signs were observed			Condition of young		
	Cornified cells in smear (25% or more)	Abnormal blood in smear	Wt loss by mother	No. dead or resorb.	No. living	Mal-formed
22	—	—	19	0	11	No
22	—	—	—	2	7	Yes
22	—	—	11	2	8	Yes
20	6–16, 18, 20	18, 20	14	all		
19	7–14, 17, 18	10, 11, 16, 19	—	3	6	Yes
19	—	19	14	4	8	Yes
19	19	15, 17, 18	15, 18, 19	all		
19	7–18	10, 11, 19	15, 16	all		
18	4–11, 13, 14, 17, 18	15, 17, 18	13	all		
17	—	15, 16, 17	12, 13, 15	7	5	Yes
16	13–16	15	12, 14	all		
16	8, 10, 11	11, 15	14, 15, 16	all		
15	8–15	15	—	all		
15	1–13	11	14, 15	all		
15	9–11, 13	15	11	all		
15	4, 9, 14	15	13, 15	5	5	Yes
Controls						
22	—	—	12	3	9	No
22	—	15, 16	—	0	3	No
22	13, 14	17–20	13	4	4	No
22	—	—	—	0	14	No
22	—	—	—	0	7	No
20	—	—	—	1	13	No
17	—	—	13	0	11	No

by the mother) the future course of pregnancy might be determined with some degree of accuracy.

Fetal death. Of the 36 females raised until maturity on the preparatory diet 34 mated as indicated by sperm in the vaginal smear, but only 30 were later verified to have become pregnant. The first 16 pregnancies, after being transferred to the more highly purified diet, were allowed to continue to term or until there was definite evidence of prior resorption of some of the fetuses. Thus, this group provided a check on the reliability of the proposed criteria to be used in predicting intra-uterine destruction of the fetuses. Only 3 of these 16 pregnancies were sufficiently uneventful that they were allowed to proceed to the 22nd day of gestation (Table I). Thirteen were interrupted earlier by killing the mother when it was judged unlikely that any of the conceptuses would survive until term. Such judgment was made after at least two, and usually all, of the following signs became positive: 1) 25% or more of cornified cells in the vaginal smear during pregnancy; 2) blood in the vaginal smear in excess of, or at times other than, that characteristic of the "placental sign", which appears normally as a trace between the 11th and 15th days; and 3) a loss of weight by the mother, particularly after the 10th day.

It is apparent from the data in Table I that these criteria were of value in indicating the fate of the fetuses. In the absence of cornified cells and abnormal quantities of blood in the vaginal smear, a majority of the fetuses may be expected to survive until the 22nd day, as was observed in 3 vitamin A deficient and a large percentage of the control mothers. Conversely, cornified cells, to the extent of 25% or more were observed at one time or another in 11 of 13 females whose pregnancy was later interrupted. All fetuses in 9 and several in the remaining 2 pregnancies were dead or already undergoing resorption. Two A deficient females, whose pregnancy was interrupted for other reasons, failed to show as much as 25% of cornified

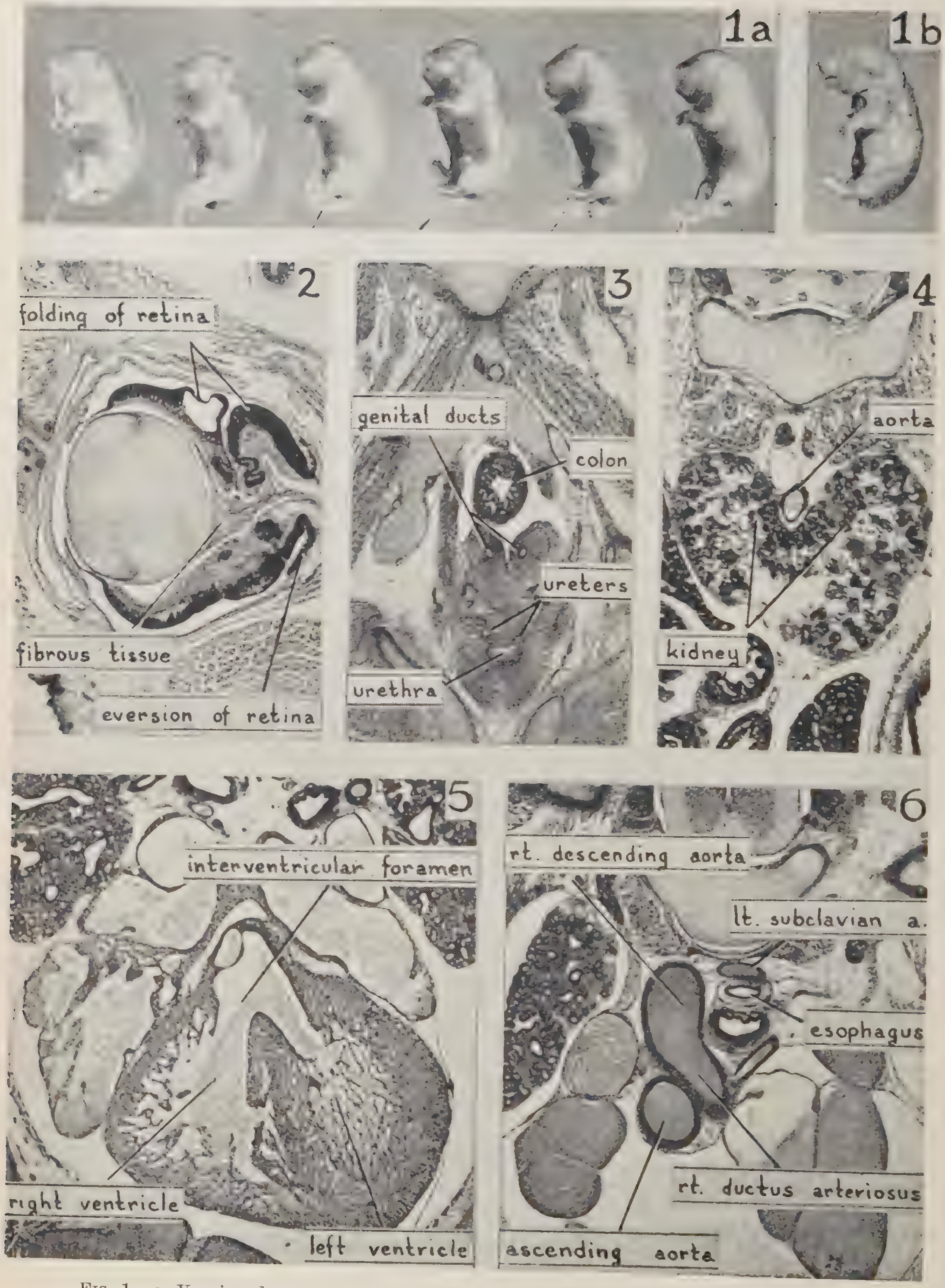

FIG. 1. a. Varying degrees of edema in young rats removed on the 22nd day of gestation from a mother fed vitamin A deficient diets. b. A normal newborn rat from a mother fed vitamin A deficient diets but supplemented with 150 μg of carotene per week.

FIG. 2. Abnormal eye of a 22-day rat fetus, from a vitamin A deficient mother. The nervous layer of the retina is deeply folded, contains cysts, and is everted on to the pigmented layer in the region of the optic stalk and along the persistent choroid fissure. The vitreous chamber is reduced to a small cone-shaped volume which is filled with fibrous connective tissue and remnants of the hyaloid artery. Retinal coloboma was present but is not apparent in this section. × 25.

FIG. 3. Ectopic openings of ureters in a 19-day rat fetus from an A deficient mother. The left ureter is seen to terminate in the urethra, at some distance below its usual termination at the base of the bladder (normally attained by the 17th day). The right ureter emptied at an even lower level in the urethra. × 25.

FIG. 4. Horseshoe kidney in a 19-day rat fetus from an A deficient mother. × 25.

FIG. 5. Persisting interventricular foramen in the heart of a 19-day rat fetus from an A deficient mother. The foramen is normally closed by the 17th day of gestation. × 25.

FIG. 6. Right-sided aorta and right-sided ductus arteriosus in a 19-day rat fetus from an A deficient mother. The left subclavian artery is also anomalous in that it arises from the descending aorta and passes behind the esophagus to reach the left shoulder. × 25.

cells at any time during pregnancy; and both yielded some living young.

Blood in the vaginal smear, in excess of or at times other than that seen during the placental sign, was noted in all of the pregnancies which were later interrupted (Table I). This would seem to be in itself an excellent indicator of fetal destruction, were it not for the fact that a subjective estimate of the quantity of blood is necessary when it appears at the same time as the placental sign. A further limitation of this criterion was noted in that, prior to the 15th day, death and partial resorption of all fetuses often occurred before excessive blood was present in the vagina. On the other hand, when resorption of some conceptuses began during the last third of pregnancy, the quantity of abnormal blood was roughly proportional to the extent of fetal destruction. Two controls showed what was judged to be abnormal blood, and in both cases there was evidence that resorption of some fetuses had begun late in pregnancy.

Loss of weight in the mother was recorded for all but 2 of the pregnancies interrupted prematurely. Also, it was observed in 3 of the controls. The degree of loss was of more significance than the mere fact that a female failed to gain weight. Both the degree and rate of loss appeared to be determined by the number of fetuses undergoing resorption at any one time, as well as by the size of the fetuses at the time of their death.

Thus, of the 3 criteria used in following the course of pregnancy in vitamin A deficient females, no one was found to entirely reliable as an indicator of fetal destruction. However, when all 3 of these signs became positive during pregnancy, several if not all of the fetuses of that pregnancy were later found to have undergone intra-uterine destruction. Pregnancies interrupted after the appearance of only 2 of the criteria, despite the fact that they were not interrupted immediately, generally yielded some living young.

In an additional group of 14 vitamin A deficient females (not included in Table I) pregnancy was interrupted immediately when the second of any 2 of the signs described above became positive. Accordingly, all of these were killed between the 11th and 15th days. Of this group, 12 females yielded intact embryos, and all but one of these contained more intact than resorbed embryos. This procedure, therefore, proved successful as a means of recognizing impending destruction of the conceptuses, thereby providing embryos and placentae which, although doubtless affected by maternal vitamin A deficiency, had not yet begun disintegration. This material is being prepared for histologic and histo-chemical study of the early stages of abnormality in the embryos and of possible associated changes in the placentae.

Malformation of the surviving fetuses. Nineteen of the vitamin A deficient females mentioned in the preceding section yielded offspring suitable for histologic study, but only the fetuses of 15 days gestational age or older have yet been examined for malformations. Of the 7 "litters" comprising the latter group (Table I), all but one contained malformed fetuses. The one litter of normal offspring was from one of the 3 pregnancies which proceeded to term without appreciable indications of fetal destruction. No malformations or other abnormalities were found in the offspring of the control animals, which were fed the same diets and subjected to the same procedures as the A deficient mothers except that the controls were given 150 micrograms of carotene per week, both prior to and during pregnancy.

From the 6 abnormal litters older than the 14th day, at least 2 and in most cases all of the young have been serially sectioned and studied. Abnormality of the older fetuses was evident before sectioning by external appearance alone: the characteristic edema of head, neck and thorax was apparent to a variable degree in all (Fig. 1 a and b). Hemorrhage in the region of the eye and prenatal opening of the lids were occasionally noted.

Warkany and collaborators(7) have reported that ocular abnormality was the most prevalent type of developmental defect resulting from material vitamin A deficiency. This was substantiated by microscopic study of animals from the 6 abnormal litters listed in Table I. All such animals examined had ocular anomalies, the more common of which were: folding and eversion of the retina, presence of a mass of fibrous connective tissue in the much reduced vitreous chamber, persistence of the choroidal fissure (coloboma), and absence of the ciliary body (Fig. 2).

Genito-urinary organs also were frequently found to be the sites of malformation in earlier observations.(8) The same types of defects have again been encountered in a similar percentage of animals. Among the commoner abnormalities in both instances were hypoplasia of renal parenchyma, renal ectopia, ectopic ureteric openings (Fig. 3), incomplete development of Müllerian ducts, abnormal retention of heterologous genital ducts in both sexes, aplasia of vagina and faulty differentiation of the urogenital sinus. Less frequent abnormalities were horseshoe kidneys (Fig. 4), cryptorchidism, hypospadias, and atresia of genital ducts and ureters.

Cardiovascular anomalies were found in 44% of a selected group of fetuses and newborns from vitamin A deficient mothers in a previous study.(9) In the present, somewhat smaller group of animals an even higher incidence (more than 60%) of malformations of the heart and major arteries was observed. In general, the anomalies were of the same type, but a few varieties not previously seen were encountered. Failure of the interventricular septum to close was again the most common defect of the heart (Fig. 5). Variations in the aortic-arch pattern frequently observed in both instances were: distally-arising right subclavian artery, right-sided arch of aorta (Fig. 6), double arch of aorta, absence of 4th arch, absence or bilateral persistence of the ductus arteriosus, and absence of a pulmonary artery.

The occurrence of keratinizing metaplasia in epithelia of the lower genito-urinary tract of the offspring from A deficient mothers has been interpreted as indicating that such offspring had themselves experienced *in utero* a state of deficiency similar to that in the mother.(10) The same interpretation may be applied to the present group of animals, in which metaplastic keratinization was again observed in the genito-urinary tract of fetuses older than 18 days gestational age.

Summary. This report constitutes a confirmation of the original experiments of Warkany and co-workers in which congenital malformations were induced in fetal and newborn rats by maintaining the mothers on vitamin A deficient diets. In addition, a method has been standardized which permits prior recognition of imminent death and resorption of the conceptuses, thereby making it possible to collect many severely malformed fetuses that would otherwise be destroyed *in utero*. Three criteria that proved reliable in anticipating prenatal destruction of fetuses were: 1) the presence of more than 25% of cornified cells in the vaginal smear at any time during pregnancy; 2) the presence of blood in the vaginal smear in excess of or at times other than that

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9. Wilson, J. G., and Warkany, J., *Am. J. Anat.*, 1949, v85, 113.

10. Wilson, J. G., and Warkany, J., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 419.

comprising the usual placental sign; and 3) a loss of weight by the mother after the 10th day of pregnancy.

Malformations involving the eye, cardiovascular system, and genito-urinary tract were found in the fetuses or newborns from all but one of the pregnancies from which intact offspring were obtained after the 14th day of gestation.

Received October 26, 1949. P.S.E.B.M., 1949, v72.

Beef Erythrocyte Extracts Reacting with Hemagglutinins in Infectious Mononucleosis and in Horse Serum Sickness. (17544)

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In previous publications(1,2) it was demonstrated that 2 serologically active substances can be isolated from the stroma of beef erythrocytes; one reacts with the hemagglutinins that are formed in infectious mononucleosis, the other, with those formed in horse serum sickness. The stroma of beef erythrocytes was first exhaustively extracted with acetone and then with 100% ethanol at room temperature, thus removing serologically inactive substances to a large extent. In order to isolate the specific reactants the stroma residue was then extracted with boiling 100% ethanol, and subsequently, with boiling 80% ethanol. As can be seen from Table I, the 80% ethanol extract yields the so-called mononucleosis antigen (M.A.) and the extract obtained with boiling 100% ethanol, the so-called serum sickness antigen (S.S.A.). Complete and distinct separation of the two serologically active substances could not be accomplished. The fractions do not correspond to pure haptens. However, on the basis of serological activity it could be assumed that the individual fractions were only 2-6% contaminated by the heterogenic fractions. Since the individual fractions are serologically highly active it was thought probable that they might demonstrate significant chemical differences which might point to the nature of the individual haptens. On the basis of previous investigations the nature of these haptens was disclosed only to the extent that they were found to be thermostable, not digestible by trypsin and pepsin, and did not give reactions characteristic for proteins. This report deals with the chemical analysis of the serologically active fractions of the beef erythrocytes. In addition, the glucose and glucosamine content after hydrolysis is presented.

Methods. 1. The C–, H– and N– determinations were done according to Pregl's method of quantitative elementary microanalysis.

2. Phosphorus was determined as ammoniumphosphomolybdate.

3. The reducing power was determined according to the Hagedorn-Jensen method and calculated for the glucose equivalent. The fractions were hydrolyzed at 100°C with 2 N HCl for 2 hours, then neutralized.

4. The glucosamine determination was done according to the method of Boyer and Fuerth as modified by Brunius.(3) 25-75 mg of dry substance were suspended in 7.5 ml of 4 N HCl and diluted with distilled water to 10 ml. This was hydrolyzed at 100°C in a sealed test tube for 5 hours. After filtration, whenever necessary, color clarification was effected by charcoal adsorption. This solution was carried to dryness over KOH and redissolved in 2 ml of distilled water. This, after

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* This work was carried out by grant in aid from the Roche Research Fund, Basel, Switzerland.

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2. Tomcsik, J., and Schwarzweiss, H., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 562.

3. Brunius, F. E., *Chemical Studies on the True Forssman Hapten, etc.*, A. B. Fahlcrantz Boktryckeri, Stockholm, 1936, p. 141-144.

TABLE I.
Extracts of Beef Stroma and Their Inhibition Titer Toward Sheep Cell Agglutinins.

Extraction	Extracted substance in %	Inhibition titer toward sheep hemagglutinins in	
		Inf. mononucl.	Serum sickness
Acetone, room temp.	8.6	0	0
Ethanol, 100% room temp.	12.6	250	16,000
’’ 100% boiling	0.7	16,000	*250,000*
’’ 80% ’’	5.1	*1,000,000*	16,000

TABLE II.
Chemical Analysis of the Ethanol Extracts of Beef Stroma.

% of	Ethanol, 100% room temp.	Ethanol, 100% boiling	Ethanol, 80% boiling
C	68.13	67.16	56.61
H	11.88	10.54	7.74
N	3.04	3.47	8.45
P	0.35	0.37	2.44
Reducing sugar	2.7	8.31	17.4
Glucosamine	1.76	4.03	8.56

addition of acetylacetone and N Na_2CO_3, was heated on the water bath and acidified with acetic acid; Ehrlich's reagent was added, air was blown through, and the volume was diluted to 10 ml with glacial acetic acid. The intensity of the red color obtained was determined with a Klett-Summerson photoelectric colorimeter. The calibration curve within the range of 0.2-1.4 mg of glucosamine, was approximately a straight line. The glucosamine content of the unknown solution was interpolated according to this calibration curve.

Results. The serological activities of the various beef stroma fractions are shown in Table I. The results of their chemical analyses (average values of several determinations) are given in Table II.

The analytical values of the S.S.A. containing substance, (extracted with boiling 100% ethanol), shows a higher content of reducing sugar and glucosamine than the slightly active fraction obtained with 100% ethanol at room temperature. Significantly different analytical values were found in the M.A. containing substance which had been extracted with 80% ethanol; here the C– and H– content were lower, the content of N– and P–, as well as that of the reducing sugar and of glucosamine content, were considerably higher. After further purification(1) of the M.A. extract, both its serological activity and reducing properties were somewhat increased (reducing sugar 19.4%). The inactive acetone extract was tested only for reducing substances after the Hagedorn-Jensen method (reducing sugar 0.23%).

The chemical nature of the serologically active M.A. and S.S.A. containing fractions of the beef erythrocytes cannot be determined because of the lack of purity of the preparations. However, it may be inferred that both substances, especially the so-called mononucleosis antigen, belong to the same group of substances as the human A substance and the Forssman antigen, and that glucose or glucosamine play an important role in the hapten structure.

Received November 14, 1949. P.S.E.B.M., 1949, v72.

Vitamin D Intake and Susceptibility of Mice to Experimental Swine Influenza Virus Infection.*† (17545)

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(Introduced by Jerome T. Syverton)

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Considerable interest has been manifested in recent years in the influence of nutrition on experimental infection. Reviews on the subject have been written by Aycock and Lutman,(1) Schneider,(2) and more recently by Clark *et al.*(3) More specifically related to the results presented in this paper is the work of Sprunt(4) who demonstrated that mice, maintained on low protein diets, showed, after injection with methionine, an increased susceptibility to swine influenza virus infection. Similarly, Midzuno(5) was able to produce influenza in rats by maintaining them on diets deficient in vitamin D. He also carried out similar experiments with avitaminotic D mice. In this paper results are presented which indicate that mice fed diets low in vitamin D are more susceptible to experimental swine influenza virus infection than are mice that receive vitamin D. These findings confirm those reported by Midzuno.(5)

Materials and methods. Experimental animals. The mice were obtained by 3 successive matings of 75 females and 35 males of second generation stock derived from Bittner's ZBC strain(6) with a commercial strain of Swiss mice. The breeding colony was maintained on Purina Fox Checkers and water. To assure uniformity in the ages of the test mice, all breedings for each replicate of the experiment were carried out within a period of 7 days. Mice were weaned at 21 days, and then placed on the experimental diets for 28 days. Thus, at the time of inoculation with swine influenza virus, the mice were at least 49 days old and not more than 56 days old.

Experimental diets. The basal diet was prepared by hand mixing all the ingredients listed in Table I, except the brewers' yeast, which was added later as the vehicle for vitamin D. The source of vitamin D was Fleischman's FIDY yeast type 9F containing 9000 U.S.P. units of vitamin D per gram. It was premixed with Fleischman's 2019 dry brewers' yeast. The desired levels of vitamin D were obtained by adding different quantities of the mixture to the diets. For the first experiment, the following amounts of vitamin D were incorporated into the diets: 0, 2.5, 5, 10, 50, 100, 250, 500 and 1000 U.S.P. units per 100 g of feed. In the second replicate experiment, 0, 10, 50, 100 and 500 U.S.P. units per 100 g of feed were used and in the third replicate experiment, 0, 2.5, 5, 10 and 100 units were fed. The diets were fed for 38 days including the 10 days following intranasal instillation of swine influenza virus into 593 test mice. Controls consisted of 123 mice also kept on the different diets for 38 days.

Mouse infectivity. The test virus was swine influenza virus (Shope 15) adapted to both eggs and mice. The mice were infected intranasally with 0.05 cc of a virus suspension

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* Aided by grants from the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y., and the Hormel Foundation, Austin, Minn.

† Publication No. 45 of the Hormel Institute, and Paper No. 2513, Scientific Journal Series, Minnesota Agricultural Experiment Station.

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TABLE I.
Basal Experimental Diet.

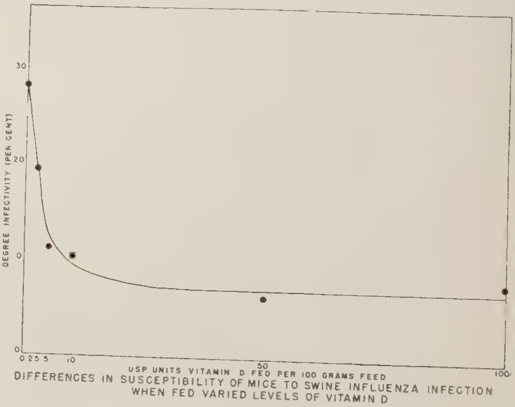
Corn starch				72%
Casein (Borden 52 edible)				18%
Corn oil				4%
Salts				4%
$CaHPO_4$	46.3%	$FeSO_4$	2.10	
$CaCO_3$	16.9	KI_4	.22	
NaCl	13.5	ZnO	.04	
$MgCO_3$	8.5	$CuSO_{14}$	.02	
KCl	6.7	$MnCl_2$	.02	
KH_2PO_4	5.7			
Vitamins				Trace
Vit. A	900 U.S.P. units	100 g diet		
Choline chloride	200 mg	,,		
Inositol	21.6 mg	,,		
Pantothenic acid	4.4 mg	,,		
Vit. E	4.0 mg	,,		
Niacin	4.0 mg	,,		
Paraminobenzoic acid	4.0 mg	,,		
Riboflavin	1.6 mg	,,		
Thiamine hydrochloride	0.8 mg	,,		
Pyridoxine hydrochloride	0.8 mg	,,		
Brewers' yeast				2%
				100%

diluted in buffered saline to approximately the minimum infective dose$_{50}$. By the method of Reed and Muench,(7) the dilution for the ID$_{50}$ was found to be $10^{5.4}$ for mice on a diet containing 100 U.S.P. units of vitamin D. In the first and third experiments, a dilution of $10^{5.7}$ of allantoic fluid was used, obtained from embryonated eggs harvested 48 hours after inoculation with 10^3 dilution of stock virus. The dilution of virus used for infecting mice was determined by preliminary tests on a small number of mice on a diet containing 100 U.S.P. units of vitamin D. In the second experiment, the preliminary virus titration was misleading, resulting in a $10^{7.0}$ dilution for inoculation of the mice.

The method for determining the extent of mouse infectivity was essentially that of Horsfall(8) and of Lauffer and Miller.(9) Mice dying of influenza within the 10-day test period were each given a score of 4, and those that showed consolidation of ¾, ½, ¼, or a trace of the lung area at autopsy on the 10th day were given scores of 3, 2, 1, and 0.5, respectively. The final positive scores differed from those of Lauffer and Miller in that the total score for each test group was divided by four times the number of mice rather than by the total number of mice. This variation made it possible to represent infectivity as a percental number of the maximal possible score that would result from death of all inoculated mice.

Results. Fig. 1 shows graphically the relative reactivity of the mice which had been maintained on graded doses of vitamin D to experimental infection with swine influenza virus. This curve, based on the infection of 593 mice, indicates clearly that mice fed for 28 days on a diet containing essentially no vitamin D were more susceptible to experimental infection than mice receiving some vitamin D in their diet. As little as 2.5 U.S.P. units of vitamin D per 100 g of diet afforded definite protection, and 5.0 units gave nearly maximal protection. The differences in relative infectivity of mice on the O diet as compared to those on the 2.5 diet, and the mice on the 2.5 diet as compared to those on the 5.0 diet were significant ($\chi^2 = 9.36$ and 7.74, df = 1, P less than 1%). There were no significant differences between the other adjacent points on Fig. 1. Points for 250, 500, and 1000 units of vitamin D are not shown on the curve, as they would only extend the curve parallel to the abscissa.

The control mice which were fed the same



DIFFERENCES IN SUSCEPTIBILITY OF MICE TO SWINE INFLUENZA INFECTION WHEN FED VARIED LEVELS OF VITAMIN D

FIG. 1.

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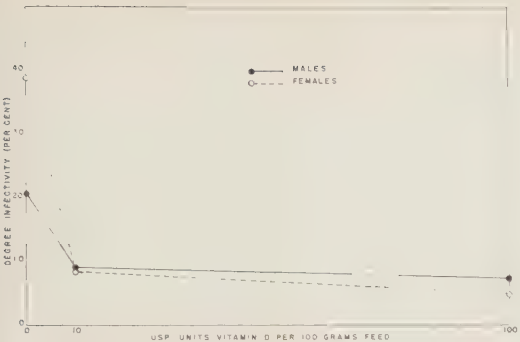

RELATIVE INFECTIVITY OF SWINE INFLUENZA VIRUS FOR MALE & FEMALE MICE ON VARIED VITAMIN D DIETS

Fig. 2.

graded amounts of vitamin D as the test animals but which were given no virus failed to show evidence of pulmonary consolidation, although the lungs of mice on the lower levels of vitamin D appeared hyperemic. Evidence for vitamin D deficiency was not observed. The mean weight for all mice at the time of weaning was 9.3 g and at the time of infection 22.4 g, the gain in weight having been progressive for all mice to the time of inoculation.

The evidence presented graphically in Fig. 2 shows that susceptibility to infection reflects a sexual difference, as well as a relationship to the dosage of vitamin D. The females were more susceptible than males on diets with no added vitamin D. The differences between males and females on diets containing 10 and 100 U.S.P. units of vitamin D were negligible. Statistically, females on an avitaminotic D diet were significantly more susceptible than males on the same diet, or than females on a diet containing 10 units of vitamin D per 100 g of feed ($\chi^2 = 12.6$ and 43.4, df = 1, P less than 0.1%). Likewise males on the diet containing no added vitamin D were more susceptible than males on the 10 units diet ($\chi^2 = 8.6$, df = 1, P less than 1%).

Summary. Experimental evidence is presented which indicates that mice fed diets containing little or no vitamin D for 28 days after weaning showed increased susceptibility to experimental swine influenza virus infection. Females were more susceptible than males.

Received November 15, 1949. P.S.E.B.M., 1949, v72.

Histochemical Localization of True Lipase.* (17546)

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In the course of experiments on the histochemical specificity of esterases (lipases) 35 different water-soluble substrates[†] were tried. Chemically, the substrates were long-chained (C_{12} to C_{18}) fatty acid esters of polyglycols or of sorbitan in which the remaining hydroxyl groups were etherified with ethylene oxide chains of various lengths. The fatty acids of these compounds were mostly saturated (lauric, palmitic, and stearic); however, a number of them contained unsaturated acids, either practically exclusively (undecylenic, oleic, and ricinoleic) or predominantly (fatty acids of linseed oil).

Experimental. Tissues of several species (man, dog, cat, guinea pig, rabbit, rhesus monkey, rat, mouse, and pigeon) were used. They were fixed in chilled acetone and embedded through acetone, alcohol, alcohol-ether, 4% collodion in alcohol-ether (12 hr) and 2 changes of chloroform, 1 hr each, in paraffin. As many as 20 different tissues were included in a single paraffin block. The in-

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[*] This work has been done under grants from the Douglas Smith Foundation for Medical Research of the University of Chicago, and from the Pathology Study Section of the U. S. Public Health Service.

[†] Obtained by courtesy of the Atlas Powder Co., Wilmington, Del., the Onyx Oil and Chemical Co., Jersey City, N.J., the Glyco Products Co., Brooklyn, N. Y., and the General Aniline and Film Corp., New York City.

cubating mixtures were prepared by adding 1 ml of a 5% stock solution of the various substrates to 50 ml of a 0.04 M tris(hydroxymethyl)aminomethane-maleate buffer(1) of pH 7.2 to 7.6, containing 0.2% of $CaCl_2$. A few crystals of camphor were added to prevent bacterial growth. Incubation time was 6 to 18 hr. The precipitates of Ca soaps were visualized by the original method.(2) Chemical controls were run by assaying the enzymatic activity of filtered homogenates of tissues according to a turbidimetric technic(1,3) The substrates were used in a concentration of 1% (somewhat less than 0.01 M, the molecular weights of the substrates being in the range of about 1200 to 1600).

Results. All substrates gave consistently good positive reactions with the exception of the undecylenic ester (CRL-16838; Atlas Powder Co.) with which only a few fair results were obtained, besides a large number of partial or complete failures. This may be explained by Ca undecylenate not being sufficiently insoluble. There was a considerable variation in the intensity of the reaction given by the various substrates. On the whole, laurates gave a more intense reaction than palmitates, and the latter a more intense one than stearates. Very long ethylene oxide side chains (as in G-2153; Atlas Powder Co.) seemed to slow down the reaction. However, the most striking differences were observed when results obtained with the use of esters of saturated fatty acids were compared with those given by unsaturated esters. The pictures published previously(4) were obtained exclusively with saturated substrates while with unsaturated substrates the localization of the reaction was greatly restricted, being invariably and intensely positive only in the pancreases of all species (especially the rat and the mouse), and in certain cells in the stomachs of some species (man, monkey, mouse). The liver of the pigeon stained rather intensely; that of man and of the guinea pig, to a much lesser degree; livers of the other species were practically negative, and so was the intestinal mucosa of most species (man, rat, mouse, rabbit); in others (monkey and pigeon) the intestinal mucosa did show some reaction but it was very much weaker than that obtained with saturated substrates. Whether these faint reactions were due to the presence of small amounts of saturated esters in all substrates or to a slight action of esterases on unsaturated ones could not be decided. They were completely abolished by the addition of 0.005 M taurocholate to the incubating mixture while the reaction in the pancreas was markedly intensified. This selective effect of bile acid on pancreatic lipase has been described previously.(5) Usually there was also some positive reaction in the intestinal contents and on the surface of villi; it appeared to be mostly extracellular and probably due to pancreatic juice or to bacterial enzyme. The kidneys of all species positive with the original technic (mouse, rat, dog, rabbit, monkey) were consistently negative when unsaturated substrates were used. The best substrates to show this restricted reaction were Tween 80, G-2144, G-9446N and G-7627DJ (oleates); G-6486 (ricinoleate) and G-9926T (linseed), all of the Atlas Powder Co.; Neutronyx R (oleate) of the Onyx Oil and Chemical Co., and Antarox B290 (ricinoleate) of the General Aniline and Film Corp. Ricinoleates, as a rule, gave distinctly weaker but even more selective reactions than either the oleates or the linseed ester. It should be remarked that the unsaturated substrates do not seem to diffuse through collodion membranes; therefore, if they are to be used, the slides must not be collodionized; in fact, the celloidin from the embedding procedure should be removed with alcohol-ether.

Chemical assays were in complete agreement with the histochemical findings. Table I shows the activity of hepatic and pancreatic extracts towards a number of substrates; it is obvious that while the pancreatic enzyme attacks all of them, the hepatic enzyme leaves the unsaturated ones practically untouched.

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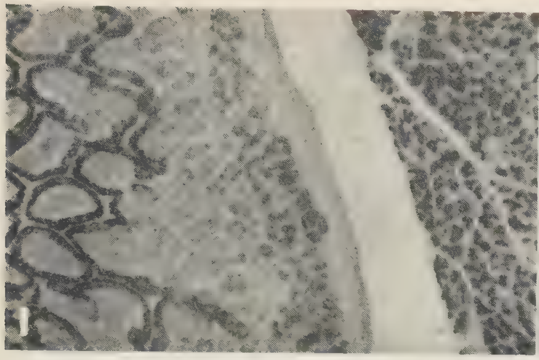

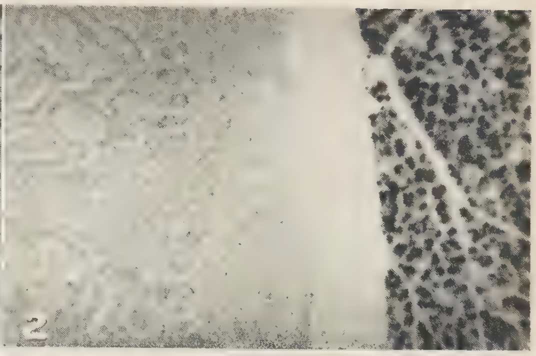

FIG. 1 (left). Pancreas and duodenum of the mouse. Substrate, Tween 60. Intense reaction in both the pancreas and the intestine.

FIG. 2 (right). Next section of the same block. Substrate, Tween 80. Reaction positive in the pancreas only; no reaction in intestine.

Comment. The differences between hepatic and pancreatic esterase have long been recognized, the most conspicuous feature of the pancreatic enzyme being its ability to hydrolyze any fatty acid ester, true fats included, while the action of hepatic enzyme is limited to simpler esters. These differences have been analyzed by numerous workers, and a number of important features have been emphasized such as the different nature of their substrates (aliphatic *vs.* aromatic;(6) straight *vs.* branched chains;(6) optical isomerism;(7,8) importance of the chain length of the fatty acid;(6,9) differences in patterns of hydrolysis of short-chained fatty acid esters of nitrophenol),(10) and differences in the effects of activators and inhibitors.(5) The ability of the pancreatic enzyme to attack true fats has led to the usage of designating it as lipase, while the hepatic enzyme is usually referred to as esterase, although both types of enzymes are sometimes included in either of the terms mentioned. To avoid confusion, it is suggested that esterase(s) hydrolyzing true fats (as the pancreatic and gastric enzyme) be called true lipase(s). The results presented add a new distinctive feature for the characterization of true lipases, namely, their unique ability to hydrolyze esters of unsaturated fatty acids at a fast rate.

Some unsaturated fatty acids are known to be essential in the nutrition of the rat. (11,12) In cystic fibrosis of the pancreas of children it has been shown(13) that the iodine number of serum lipids is lowered, thus indicating the possibility of a selectively poorer utilization of unsaturated fats as compared to that of saturated ones. Whether the low iodine numbers of serum lipids in infantile eczema(14-16) are also due to pancreatic hypofunction is not known. The dependence of some of the symptoms on a deficiency of unsaturated fatty acids is a moot question because it has never been ascertained that the latter are essential in human nutrition. However, should they prove to be so, the results of the experiments presented would indicate

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TABLE I.
Hydrolysis of Water-soluble Fatty Acid Esters by Hepatic and Pancreatic Esterase.
Buffered substrate, 10 ml; 4% tissue extract (rat liver and pancreas), 0.5 ml. Activities expressed in micromoles of fatty acid liberated in 1 hr.

	Tween 20 (lauric)	Tween 40 (palmitic)	Tween 60 (stearic)	CRL-16838 (undecylenic)	G-2144 (oleic)	G-6486T (ricinoleic)	G-9926T (linseed)
Liver	6.6	3.8	3.3	0.4	0.4	0.0	0.5
Pancreas	40.0	19.6	10.4	16.8	16.6	3.0	19.2

that in attempts at therapy they must be administered pre-hydrolyzed, as soaps and not as fats, since hydrolysis of the latter in the intestinal tract is likely to be inadequate. It should be remarked that in the human intestine even esterase reaction is weak.

Summary. Water-soluble unsaturated fatty acid esters are hydrolyzed almost exclusively by the pancreatic type of esterase (true lipase) while similar esters of saturated fatty acids are readily attacked by esterases of both the hepatic and pancreatic type. This difference is utilized in a technic for the histochemical localization of true lipase activity. The findings presented may have implications in human pathology.

Received Nov. 21, 1949. P.S.E.B.M., 1949, v72.

Metabolism of the Steroid Hormones. Conversion of Desoxycorticosterone to Glycogenic Material *In vitro.*[*] (17547)

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Recently, Hechter *et al.*(1) have reported the conversion of desoxycorticosterone to corticosterone by the isolated adrenal gland using a perfusion technic. We have been able to demonstrate the conversion of desoxycorticosterone into material possessing glycogenic properties with adrenal slices and an adrenal homogenate preparation.

Desoxycorticosterone,[†] either as the free compound or the glucoside, was incubated for 6 hours at 38°C with adrenal slices, homogenates, or a combination of both. In Experiments A, B, C (Table I) where adrenal slices were used, 50 mg of steroid were dissolved or suspended in a total volume of 25 ml containing 0.01 M glucose, 0.062 M sodium chloride, and 0.02 M sodium phosphate buffer (pH 7.4). The gas phase was air. In Experiment D, 50 mg of steroid was contained in a total of 25 ml of media containing 5.0 ml of a 10% boiled adrenal cortical extract in saline, 0.01 M sodium fumerate, 0.062 M sodium chloride, 0.025 M potassium chloride, and 0.004 M magnesium sulfate. The gas phase was air.

At the end of incubation, 400 ml of acetone were added and the mixture placed in the cold overnight. The precipitated tissue was filtered with suction, ground, and extracted 3 times with hot acetone. Tissue slices were cut into small pieces with a scissors before grind-

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[*] Supported in part by a grant (RG 1050) from the Division of Grants and Fellowships, National Institutes of Health, U. S. Public Health Service.

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[†] The desoxycorticosterone and desoxycorticosterone glucoside were generously supplied by Ciba Pharmaceutical Products, Inc.

ing. The combined acetone filtrates were evaporated to an aqueous sludge, 25 ml of distilled water added, and brought to pH 7.0. This aqueous residue was extracted 3 times with ethylene dichloride. The ethylene dichloride extract was concentrated to dryness and taken up in corn oil for bioassay by a mouse glycogen method.(2) The results are expressed in 11-dehydrocorticosterone equivalent.

The results are summarized in Table I. Five grams of adrenal tissue incubated without steroid yielded 0.2 mg equivalent of 11-dehydrocorticosterone or less. For the calculation of net amount of glycogenic material formed the value of 0.04 mg of 11-dehydrocorticosterone per g of tissue was used as the amount of activity material contributed by the tissue as such. The unreacted desoxycorticosterone contributed a negligible amount of glycogenic activity.

Incubation of desoxycorticosterone either with slices or homogenate resulted in the formation of glycogenic material. The yields were higher when the glucoside was used. Incubation of the glucoside with the homogenate gave a higher yield of glycogenic material than when slices were employed (Table I).

Since glycogenic activity in the adrenalectomized mouse is associated with steroids having oxygen at carbon 11, it is presumed that the enzyme system is concerned with the oxidation of carbon 11 to either a carbonyl or an alcohol grouping.

TABLE I.
Conversion of Desoxycorticosterone to Glycogenic Material.

Exp.	Amt of desoxy-corticosterone equivalent added, mg	Adrenal tissue added, wet wt, g	No. of mice used	Equivalent of 11 dehydro-corticosterone found, mg	Net amt of glycogenic material formed calculated as 11 dehydro corticosterone, mg	Conversion, %
	0	5 (Slices)	6	< .20	—	—
	0	5 ,,	5	.20	—	—
	0	5 (Homogenate)	5	< .20	—	—
A	100 (Free)	4.5 (Slices) 1.5 (Homogenate)	7	3.84	3.60	3.6
B	67.0 (Glucoside)*	4.5 (Slices) 1.5 (Homogenate)	7	4.40	4.16	6.2
C	33.5 ,, *	5 (Slices)	10	1.92	1.72	5.1
D	33.5 ,, *	3.5 (Homogenate)	10	3.89	3.71	11.1
	33.5 ,,	—	10	< .3		

* This preparation was a 1% solution of the glucoside containing 10% glucose and 10% acetamide.

Summary. Incubation of desoxycorticosterone either as the free compound or as the glucoside with adrenal slices or homogenate resulted in the formation of glycogenic material.

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Received November 18, 1949. P.S.E.B.M., 1949, v72.

Influence of Desoxycorticosterone on Cesium Excretion in Adrenalectomized Rats.[*] (17548)

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Adrenal cortical steroids are known to influence the metabolism of sodium and potassium, both members of Group I of the periodic table. We have studied the possible influence of one of the adrenal cortical hormones, desoxycorticosterone, on the excretion of administered cesium, the heaviest member of Group I.

The method employed has been described previously for radio-sodium(1) and for radio-potassium(2) and need only be mentioned briefly here. Male rats purchased from Holtzman (Madison, Wis.) were bilaterally adrenalectomized. Twenty-four hours after operation the animals were injected subcutaneously with 2 different doses of desoxycorticosterone[†] dissolved in 0.25 cc of corn oil. Control rats received only 0.25 cc of oil. One hour after receiving the hormone the rats were injected with 2 cc of an aqueous Cs_{134}[‡] solution containing 10.3 μg of cesium carbonate. The rats were immediately placed in glass metabolism cages and the urine collected directly into ointment tins for a period of 6 hours. The urines were evaporated to dryness and radiocesium determined as previously described for radiosodium.(1) The cesium excretion for the experimental period was expressed as the percentage of the material administered.

Table I lists the results which indicate an excretion of 5.20 ± 0.75% of the administered cesium within the 6 hour period. The administration of 25 and 250 μg of desoxycorticosterone had no significant influence on the cesium excretion. As little as one microgram of the hormone caused a significant retention of sodium(1) and 10 μg caused a significantly increased excretion of potassium.(2)

Summary. Desoxycorticosterone did not influence the excretion of administered radiocesium in adrenalectomized rats. Under specific conditions of the test 5.20% ± 0.75% of the administered radiocesium was excreted in the urine within the first 6 hours.

Received November 18, 1949. P.S.E.B.M., 1949, v72.

TABLE I.
Influence of Desoxycorticosterone on Cesium Excretion in Adrenalectomized Rats.
14 rats in each experiment.

Desoxycorticosterone, μg	Mean body wt, g ± S.E.	Mean excretion of Cs^{134}, % ± S.E.	Significance of difference, "t"
0	144 ± 2	5.20 ± .75	—
25	138 ± 2	4.28 ± .45	1.03
250	138 ± 3	4.87 ± .73	0.31

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[*] Supported in part by a research grant (RG 234) from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service, and a grant from Sharp and Dohme, Inc.

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[†] The desoxycorticosterone was kindly supplied by Ciba Pharmaceutical Products, Inc.

[‡] The radiocesium was obtained from Monsanto Chemical Co. through the U. S. Atomic Energy Commission.

Bacterial Activators (*Lysokinases*) of the Fibrinolytic Enzyme System of Serum.* (17549)

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In recent years, revived interest in a proteolytic enzyme system of the blood has resulted in new data and some difficulties as to terminology.(1,2)

In view of the practical importance of *fibrinolytic* phenomena, we have accepted the "fibrinolysin" rather than the "tryptase" or "plasmin" nomenclatures. A brief summary of these terminologies and our abbreviations follows: 1. *Lysin* or fibrinolysin (tryptase, plasmin): the active proteolytic (fibrinolytic) enzyme; 2. *Antilysin* or antifibrinolysin (antitryptase, antiplasmin): inhibitor of lysin; 3. *Prolysin* or profibrinolysin (tryptogen, plasminogen): the inactive precursor of lysin; 4. *Lysokinase* (tryptokinase): any activator of prolysin, *e.g.* streptokinase,(3) staphylokinase,(4) fibrinokinase;(5) 5. *Antilysokinase* (antitryptokinase): inhibitor(s) of lysokinase(s), *e.g.* antistreptokinase.(6)

The present study concerns methods and results of an extensive survey of available bacterial types for *lysokinase* activity. Earlier studies(3,7) showed that streptokinase specifically activates only human prolysin, while staphylokinase affects prolysin from human, dog, guinea pig, and rabbit sera but not other species tested.(8) This further search for new bacterial lysokinases and particularly for an activator of bovine prolysin has been somewhat discouraging.

Materials. Thrombin. Upjohn's bovine thrombin (courtesy Dr. J. T. Correll), 20 units per cc aqueous (sterile) solution. *Fibrinogen*: 1% aqueous solution of Armour's bovine "Fibrinogen," pH = 7.0 ± 0.2, sterilized by Seitz filtration. *Prolysin preparations*: A. For Survey Tests—(1) Human Plasma Fraction–I† (courtesy Dr. J. T. Edsall), 2% aqueous (sterile) solution, containing fibrinogen as well as prolysin; (2) Dog and Bovine Sera, sterilized by Seitz filtration: B. For Lysokinase Tests—(3) Human, Dog, and Bovine I Serum Fractions, prepared from pooled sera, at 0°C, by precipitation at 25% concentration of ethanol, the deposit after one hour being recovered by centrifugation and redissolved in one-half the original serum volume of borate buffer(7) (pH = 7.75) and stored frozen at –20°C; (4) Bovine II, a fraction prepared by 33% saturation of serum with $(NH_4)_2SO_4$, at 1°C, as previously described.(7)

Bacterial cultures. Stock cultures from the Bacteriology Laboratory of the University of North Carolina were obtained through the courtesy of Drs. D. A. MacPherson and F. L. Rights. The β-hemolytic streptococcus (No. 98) control culture was obtained from Dr. Maxwell Finland of the Thorndike Memorial Laboratory (Boston). Additional cultures isolated from bovine sources were made available through the courtesy of Dr. L. T. Giltner, U. S. Dept. of Agriculture, and Dr. H. E.

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* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

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† This product was developed from blood, collected by the American Red Cross, by the Department of Physical Chemistry, Harvard Medical School, under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

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Stock medium for survey tests. "Yeast peptone," containing yeast extract (1%), tryptose (2%), dextrose (0.2%), NaCl (1.6%), pH = 7.6 ± 0.2, sterilized by autoclaving. This *double strength* stock medium was diluted by the other materials of the test mixtures (see below). *Un*inoculated mixtures of medium plus fibrin clots, with and without added prolysin (human, dog, bovine), showed no fibrinolysis.

Methods. The first procedure was a general survey of all available bacterial cultures for fibrinolytic activity as manifested during growth in medium + fibrin clots, with and without added prolysins. Secondly, selected cultures showing positive results in the survey test, were followed up with attempted isolation of a partially purified lysokinase by the standard alcoholic precipitation of culture filtrates as used for streptokinase(9) and staphylokinase.(4)

Survey method. For aerobes, sterile fibrin clots (with nutrient medium), in 13 × 100 mm tubes, were inoculated from 24 hour broth subcultures by stabbing and seeding of surface. For anaerobes, the clots were doubled in volume and covered with paraffin. Control (no prolysin) and prolysin-containing clots were incubated at 37°C and observed after 24 and 48 hours. Bacterial tubes in which growth was not satisfactory were repeated or discarded and in those reported growth was successful. Each bacterial strain was tested in the following 4 clot mixtures: I. Control (no prolysin): 1 cc medium + 1 cc fibrinogen + 0.2 cc thrombin; II. Human: 1 cc medium + 1.5 cc Human Plasma Fraction–I (prolysin and fibrinogen) + 0.2 cc thrombin; III. Dog: 0.5 cc medium + 0.5 cc dog serum (prolysin) + 1.0 cc fibrinogen + 0.2 cc thrombin; IV. Bovine: 0.5 cc medium + 0.5 cc bovine serum (prolysin) + 1.0 cc fibrinogen + 0.2 cc thrombin.

Lysokinase study. Bacteria selected as a result of the survey test (see above) were cultured for 24 hours in 500 cc Bacto heart infusion broth and centrifuged subsequently to remove suspended bacteria. The selected anaerobe (*Cl. botulinus*) was grown in thioglycollate broth. The "lysokinase," in each instance, was prepared by the alcoholic precipitation method of Garner and Tillett,(9) yielding a dry powder, subsequently prepared for use as a 1% solution in borate buffer and tested for activation of human, dog, and bovine prolysins (see *Materials*), utilizing methods previously described.(7)

Results. Survey study. Seventy-seven cultures of 40 types of bacteria were satisfactorily submitted to the lysis tests of the 4 types noted under *Methods.* Some common organisms were omitted because cultures were unavailable at the time or, in some cases, because of failure to grow under the test conditions. Completely negative results (no significant lysis of any of the clots) were obtained with: *Streptococcus pyogenes* (5 strains, incl. 1 erysipelatis—but *cf.* Table I), *Strep. faecalis, Strep. acidominium* (uberis), *Strep. mitis* (viridans—3 strains—but *cf.* Table I); *Micrococcus* (Staphylococcus) *citreus, Micr. pyogenes, var. albus* (*Staph. albus*—2 strains); *Gaffleya tetragena; Neisseria catarrhalis; Corynebacterium pseudodiphtheriticum; Vibrio metschnikovii; Alcaligenes faecalis; Escherichia coli* (3 strains); *Aerobacter aerogenes* (3 strains); *Proteus vulgaris* (1 strain); *Salmonella (Eberthella) typhosa* (2 strains); *Shigella paradysenteriae* (7 strains), *Shig. sonnei* (2 strains); *Pasteurella multocida (suilla), Past. pseudotuberculosis; Brucella melitensis,*‡ *Bruc. abortus,*‡ *Bacillus megatherium; Mycobacterium phlei.*

Table I summarizes the survey tests on all bacteria which produced significant fibrinolysis in any of the clots, whether in the control (I), without prolysin, or in the presence of human (II), dog (III), or bovine (IV) prolysin. The degree of lysis is roughly quantitated 0——++++. Under *Comment* is noted the ability to liquefy gelatine, and the lack of correlation between this property and the fibrinolytic phenomena is noteworthy. Individual cultures of the same type often varied in the results of these tests. The strains asterisked in Table I were selected for the

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9. Garner, R. L., and Tillett, W. S., *J. Exp. Med.*, 1934, v60, 239.

‡ Grown both aerobically and in CO_2.

TABLE I.
Survey of Fibrinolytic (I) and Lysokinase (II-IV) Effects of Certain Bacteria (See text).

Bacterial type	I Control	II Human	III Dog	IV Bovine	Comment
**Streptococcus pyogenes* (β-hemolytic)	0	++++	0	0	No. 98 (Finland)
**Staphylococcus aureus* (hemolytic) (*Micrococcus pyogenes, var. aureus*)	0	++++	++++	0	Gelatine liq.
**Staphylococcus aureus*† (hemolytic)	0	++++	++++	0	,, ,,
,, ,,	0	++	++++	0	,, ,,
,, ,,	0	++	++++	0	,, ,,
Streptococcus pyogenes	0	++++	0	0	
,, *mitis (viridans)*	0	+++	0	0	
**Sarcina lutea*	0	++++	+	0	,, ,,
**Pseudomonas aeruginosa*	+	+	++++	+	,, ,,
**Klebsiella pneumoniae (friedlanderi)*	++	++	+++	++	
,, ,,	0	0	+++	0	
**Serratia marcescens*	++	++++	0	0	,, ,,
**Salmonella paratyphi*	0	+++	++	0	
,, ,,	0	0	+	0	
,, ,,	++	++	++	0	
,, *schottmuelleri*	++	0	+	0	
* ,, *typhimurium (aertrycke)*	+++	+	+	+	
,, ,,	++	0	++	0	
,, *choleraesuis*	+++	+	+++	0	
,, ,,	++	++	0	0	
,, *enteritidis (gaertneri)*	+	+++	0	0	
,, *anatis*†	+++	0	++	0	
**Shigella ambigua*	0	+++	0	0	
,, ,,	+	+	+	0	
Brucella (abortus) suis (in O_2)	++++	0	0	0	
,, ,, ,, (in CO_2)	++++	0	0	0	
**Bacillus subtilis*	+	0	++++	+	,, ,,
,, ,,	+	0	+++	0	,, ,,
,, ,,	++++	++	++++	+	,, ,,
Clostridium septicum	++++	++++	++++	+++	,, ,,
* ,, *botulinum*	++++	++++	++++	+++	,, ,,
,, ,,	++++	++++	++++	+	,, ,,
,, *perfringens (welchii)*	++++	++++	+++	+++	,, ,,

* Cultures subsequently selected for *lysokinase* tests.
† Bacteria isolated from *bovine* sources.

subsequent lysokinase studies (see below). The streptococcus and staphylococcus strains listed separately at the top of Table I are typical good lysokinase producers and have been used repeatedly in this laboratory for preparation of streptokinase and staphylokinase, respectively. Strains isolated from bovine sources are indicated (†).

Lysokinase study. As indicated in Table I, lysokinase studies (see *Methods*) were attempted on alcohol-precipitated material isolated from 12 bacterial strains, namely, 1. β-hemolytic *Strep. pyogenes* (human source), 2. hemolytic *Staph. aureus* (human source), 3. hemolytic *Staph. aureus* (bovine source), 4. *Sarcina lutea,* 5. *Pseudomonas aeruginosa,* 6. *Klebsiella pneumoniae* (Friedlander), 7. *Serratia marcescens,* 8. *Salmonella paratyphi,* 9. *Salm. typhimurium* (Aertrycke), 10. *Shig. ambigua,* 11. *Bacillus subtilis,* 12. *Clostridium botulinum.*

Fibrinolytic tests(7) were performed after preliminary incubation of "lysokinase" preparations with the 4 prolysins (see *Materials*: B. (3), (4)) as follows: (1) no incubation (tested immediately), (2) 20 min. at 37°C, (3) 1 hour at 29°C, (4) 24 hours at 6°C. The staphylokinase of bovine origin (hemolytic *Staph. aureus*) activated both human and dog prolysins to approximately the same lytic strength as our standard (human) staphylokinase. It did not affect bovine prolysin, however. The material from *Clostridium botulinum* (anaerobic) cultures was actively fibrinolytic but lysed clots containing prolysins in longer times than the prolysin-free control (lysis time: 15 min.) so that its action is presumably due solely to a proteolytic enzyme from the bacteria themselves. All other materials gave negative results in concentrations of 0.06-1.0%. There were insufficient materials for testing at higher concentrations.

Discussion. Omitted from these studies were a number of bacterial types, some of which were known to have been tested by earlier workers with the older "fibrinolytic" technics. Tillett(10) reviews a number of these and particularly states that the pneumococcus gives negative results. Our data clearly show that fibrinolytic activity is encountered in cultures of a wide variety of bacteria, but only in the case of certain hemolytic streptococci and staphylococci can a definite lysokinase be demonstrated. The results with *Clostridium botulinum* show the need for the prolysin-free control test. Fibrinolytic activity appears to be unrelated to the gelatine liquefaction test.

Summary. In a survey test of 77 satisfactory cultures of 40 bacterial types, fibrinolytic phenomena were encountered in 19 types (33 cultures) of bacteria tested. Only certain hemolytic streptococci and staphylococci gave positive results with a specific test for *lysokinase,* however, and these organisms were not directly fibrinolytic nor able to activate bovine profibrinolysin. *Direct* fibrinolytic activity may occur in cultures when (except in the case of *Cl. botulinum*) it is not found in the alcoholic precipitate. The failure to obtain a lysokinase in at least 6 types of bacterial cultures which gave good results in the survey test is disappointing, as is the failure to find a kinase for bovine profibrinolysin.

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Received October 4, 1949. P.S.E.B.M., 1949, v72.

A Substance in Egg Yolk Which Inhibits Deterioration of Aureomycin Activity.* (17550)

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In the treatment of chick embryos infected with psittacosis virus(1) the single dose of aureomycin required to protect 50% of infected embryos for 7-9 days was found to be one-third to one-fourth as great as the dose of chloromycetin required to produce the same effect. This finding was unexpected in view of the rapidity with which aureomycin deteriorates *in vitro* at 37°C under neutral or slightly alkaline conditions(2-6) as well as the relative stability of chloramphenicol under similar circumstances.(7-9) It appeared possible, therefore, that some factor, present in the embryonated egg, protected aureomycin from deterioration. In preliminary experiments, de-

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* Aided by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

† Senior Fellow in Virus Diseases, National Research Council.

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TABLE I.
Effect of Fertile Eggs Upon Deterioration of Aureomycin at 37°C.

Hr of incubation	Medium		
	Infusion broth	Intact egg	Homogenized egg
0	50.*	50	50
24	<12.5	25–50	25–50
48	2.0	32	16
72	—	16	8
96	2.0	—	6
144	1.0	12	4
168	0.5	12	3

* Aureomycin concentrations (μg/ml).

signed to test such a possibility, it was found that when 7-day old embryonated eggs were inoculated by the yolk sac route with aureomycin most of the aureomycin activity could still be demonstrated in the egg fluids as long as 7 days after inoculation. The presence of actively multiplying psittacine virus did not affect the persistence of the aureomycin activity. The following experiments were, therefore, performed to determine whether a substance exists in eggs which permits aureomycin to retain its potency despite prolonged incubation under conditions which usually destroy the activity of the antibiotic agent.

Methods. Aureomycin hydrochloride‡ was made up to the desired concentration in brain heart infusion broth (Difco), pH 7.2. Aureomycin levels were determined by a serial 2-fold dilution method similar to that used for penicillin(10) using separate pipettes for each dilution. Bacillus No. 5§ was the standard test organism. All test fluids were incubated at 37°C.

Experimental. Tests in Embryonated Eggs. In the first experiments it was sought to determine whether the protective factor was found only in relation to intact chick embryos. Seven-day old embryonated eggs, with an average weight of 61.0 g and an assumed average volume of 40.0 ml of fluid were used. A dose of 2.0 mg of aureomycin in 0.25 ml of broth was injected into the allantoic sac of each embryo. Control eggs received the same amount of broth containing no drug. Three uninoculated embryonated eggs were each homogenized aseptically in a Waring blendor; 2.0 mg of aureomycin in broth were added to 2 of them and a comparable volume of broth alone was added to the third. The homogenized eggs and the intact fertile eggs were then incubated. At intervals thereafter fertile eggs were homogenized and their contents of aureomycin assayed and compared with those in the eggs originally homogenized. A control tube containing 2.0 mg of aureomycin in 40 ml of broth was also included. The results appear in Table I. It is seen that both in the intact and in the homogenized embryos the rate of deterioration of aureomycin at 37° was much slower than in the broth. The protective effect was probably unrelated to differences in hydrogen ion concentration:—the pH of recently homogenized eggs, as determined with a Beckman potentiometer, was found to vary between 7.1 and 7.3; that of homogenized eggs after prolonged incubation, was 7.1-7.2, and that of the control broth was 7.2. In no case did eggs without aureomycin exert any significant effect on the growth of the test organism.

Tests with Nonfertile Eggs. Nonfertile eggs were examined for their capacity to delay the deterioration of aureomycin, and the relative potencies of yolk and albumen, in this regard, were also determined. An egg showing no evidence of fertility was homogenized in the blendor and 1.0 mg of aureomycin added to 20 ml of the emulsion. The yolk and albumen of another nonfertile egg were separated aseptically and aureomycin added to each in the same concentration. Broth and a 25% solution of human serum albumin, each containing the same concentration of aureomycin,

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‡ Supplied in crystalline form in sterile vials by Lederle Laboratories.

10. Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, v51, 386.

§ Obtained from Dr. B. M. Duggar.

TABLE II.
Effect of Nonfertile Eggs, Egg Yolk and Egg Albumen on Deterioration of Aureomycin at 37°C.

	Medium				
Hr of incubation	Infusion broth	Homogenized nonfertile egg	Egg yolk	Egg albumen	25% human serum albumin
0	64.0*	64	64	64	64.0
24	8.0	32	64	8	16.0
72	2.0	16	32	4	0.5
144	1.0	4	16	4	<0.1

* Aureomycin concentrations (μg/ml).

TABLE III.
Inhibition of Deterioration of Aureomycin at 37°C by Varying Dilutions of Egg Yolk in Broth.

	Hr of incubation		
Final dilution of egg yolk	0	24	72
1:2	32*	32	8
1:4	32	32	4
1:10	32	16	2
1:20	32	16	2
1:100	32	8	1
Broth alone	32	8	1

* Aureomycin concentrations (μg/ml).

served as controls. Aureomycin levels were determined immediately and at intervals after incubation. The results, which appear in Table II, show that the nonfertile egg also has the capacity to protect aureomycin from deterioration. Furthermore, the protective substance was found in the yolk. Thus, whereas the broth controls and the tubes containing egg and serum albumins retained but one-eighth of the original activity after 24 hours of incubation, there was no significant loss in the presence of whole egg or egg yolk alone. Even after 72 hours of incubation, when a 32-fold loss had occurred in the broth controls and a 16-fold loss in the presence of egg albumen, there was but a 4-fold deterioration in the presence of whole egg and a 2-fold deterioration in the presence of egg yolk. A 2- to 4-fold variation in activity has generally been considered to be within the limits of error of the method. The effect of dilution of egg yolk on its capacity to protect aureomycin from loss of potency is demonstrated in Table III. Egg yolks from nonfertile eggs were mixed with broth in varying proportions and sufficient aureomycin added to achieve a standard concentration of 50 μg per ml. It is seen that when egg yolk has been diluted beyond 1:4 its capacity to protect aureomycin from deterioration diminishes.

Subsequent experiments have shown that the protective substance in yolk resists heating at 60°C for 80 minutes. Studies still in progress indicate that this protective substance remains active after heating for one hour at 100°C and, under certain conditions, even after autoclaving at 120° for 15 minutes.

Discussion. The rapid rate of deterioration of aureomycin activity upon standing has been a constant source of concern to those working with this antibiotic. Stringent precautions are necessary to avoid loss of aureomycin activity in body fluids withdrawn for assay, and even then aureomycin deteriorates during the period of incubation required for assay.(3) Whether similar deterioration occurs within the body is not clear; however, significantly large amounts of injected aureomycin remain unaccounted for after assay of body fluids. The mechanism of the destruction of aureomycin is quite unknown,(6) but it is manifestly of value to seek a substance which will inhibit this destruction. It has been shown in the present studies that such a substance exists in egg yolk. Further studies on the isolation, characterization and properties of this substance are now in progress.

Summary. A substance (or substances) found in egg yolk, inhibits the deterioration of aureomycin activity which ordinarily occurs upon incubation at 37°C. The protective agent is heat stable and of sufficient concentration to permit dilution of egg yolk to 1:4 without significant diminution of aureomycin-protective action. Egg albumen and human serum albumin are not protective in the concentrations used.

Received November 18, 1949. P.S.E.B.M., 1949, v72.

Fractionation of Amino Acid Mixtures in Acetone by Means of Alkyl Acid Phosphates.* (17551)

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In a former communication(1) we described the property of solvating amino acids in dry acetone or dioxane, which is possessed by 8 organic acids, di- and trichloroacetic acids, and certain sulfonic acids. The purpose of the present communication is to state that we have found that several alkyl acid phosphates† also possess the property of solvating a dry mixture of amino acids in dry acetone. These may be used for dividing a protein hydrolysate into a series of fractions containing one to six or seven amino acids, certain of these composing a high percentage of the individual fractions. On adding ammonia to acetone solutions of amino acids formed by the aid of alkyl acid phosphates, both amino acids and the ammonium salts of the reagent-acids precipitate together. This property of alkyl acid phosphates contrasts strongly with that of many sulfonic acids, which, under these conditions, form salts with ammonia which are readily soluble in acetone, permitting the precipitated amino acids to be separated from the solvating agent. The triethylamine (T.E.A.) salts of the alkyl acid phosphates are soluble in acetone. This property permits the amino acids to be separated from the reagent by means of this base.

Individual amino acids show marked differences in solubility in an acetone solution of octyl, n-amyl, iso-amyl, n-butyl, iso-butyl, n-propyl and iso-propyl acid phosphates. Table I illustrates the contrasting solvating power of 5 reagent acids for 9 amino acids. We designate by the term proline equivalent, the ratio of the amount of a reagent necessary to dissolve one equivalent of amino acid to the amount of the same reagent necessary to dissolve one equivalent of proline.

The amino acids in a hydrolysate exert marked influence on each other's solubility in acetone-alkyl acid phosphate solutions. Much less acetone-reagent is required to dissolve a given weight of dry protein hydrolysate than is necessary to dissolve the component amino acids of the sample in the isolated state. There is, however, pronounced selectivity in the order in which certain amino acids are dissolved when successive portions of an acetone-reagent are passed through a sample of hydrolysate contained in a filtering crucible.

Table II illustrates some contrasting properties of alkyl acid phosphates in solvating power for a casein hydrolysate.‡ It also shows the differences in extent of precipitation of amino acids from their acetone-reagent-hydrolysate systems after neutralization with triethylamine, and the degree of complexity of the more difficultly soluble residues not brought into solution in our experiments.

A Procedure for Fractionation of a Protein Hydrolysate. A 10 g sample of the dry, finely ground, hydrolysate is thoroughly mixed with an equal weight of anhydrous calcium sulfate (Drierite), and the mixture is placed in a sintered glass crucible equipped with filter flask and suction connection. A test tube is placed inside the filter flask to collect the filtrate. The amino acid-Drierite mixture is thoroughly wet with the reagent (*e.g.* a 0.2 N solution of an alkyl acid phosphate in acetone), the contents of the crucible are stirred for a definite time (usually one minute), suction is applied, and the solution in the crucible is sucked through into the test tube. Another portion of the reagent is then added, and the

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* This investigation was supported in part by a grant-in-aid from Nutrition Foundation, Inc.

1. McCollum, E. V., and Rider, Agatha A., *Science*, 1948, v108, 111.

† The authors are indebted to Monsanto Chemical Co., St. Louis, Mo., through the courtesy of Mr. Paul Logue, for the samples of alkyl acid phosphates employed in this investigation.

‡ The authors are indebted to National Dairy Products Corp. Research Laboratories, Oakdale, L.I., through the courtesy of Dr. Arnold H. Johnson, for the sample of hydrochloric acid casein hydrolysate, their ''Hycase,'' a salt-free preparation.

TABLE I.
Proline Equivalents of Alkyl Acid Phosphates in 0.2 N Solutions in Acetone.

Amino acid	Octyl	n-Amyl	n-Butyl	n-Propyl	Isopropyl
Proline	1	1	1	1	1
Isoleucine	3	2	4	3	1
Ph. alanine	6	6	7	2	2
Leucine	6	4	5	13	2
HO-proline	12	7	14	5	3
Tryptophan	14	3	7	2	1
Threonine	16	5	7	4	2
Serine	42	42	19	9	11
Glutamic acid	58	70	42	23	8

TABLE II.
Solvating Power of 0.2 N Solutions of Alkyl Acid Phosphates, Precipitates Yielded on Neutralization with Triethylamine (T.E.A.), and Chromatographs of an Undissolved Residue.

Alkyl acid phosphate	% of sample of a casein hydrolysate readily soluble[‡]	% of soluble material precipitated on addition of T.E.A.	Results of chromatographing the insoluble material	Remarks
Octyl	97	85	—	
n-Amyl	45	80	—	[*]
Iso-amyl	95	75	3 spots	
n-Butyl	97	68	—	
Iso-butyl	97	80	1 spot	Tyrosine
n-Propyl	97	90	2 spots	
Iso-propyl	100	45	—	[†]

[*] The amyl acid phosphate-insoluble material has been provisionally identified as the basic amino acids, methionine, tyrosine (and possibly serine), alanine, proline, valine, and the leucines. This insoluble fraction contains all of the tyrosine. The moiety which is soluble in amyl acid phosphate, and precipitable with triethylamine, has been tentatively identified as a mixture of some of each of the basic amino acids, aspartic acid, glutamic acid (and possibly serine), phenylalanine, and/or valine, alanine, methionine, proline, and the leucines. All of the glutamic and aspartic acids are found in the soluble and precipitable material. The soluble material which remains in solution after neutralization with T.E.A. appears to contain almost all of the proline.

[†] The portion of the hydrolysate which precipitates from solution with iso-propyl acid phosphate after the reagent is neutralized with T.E.A. exhibits 6 spots when examined by ascending chromatography using 77% ethanol. These spots have been shown to be arginine, histidine, lysine, glutamic acid (and possibly serine), aspartic acid, alanine, and the leucines. Glutamic acid, lysine and arginine predominate in this mixture. Histidine and aspartic acid are present only in traces.

process of stirring and filtration by suction is repeated until the desired volume of filtrate has been collected. The apparatus is then disassembled, the test tube containing the filtrate is replaced by an empty one, the apparatus reassembled, and the process of fractionation continued. The size of the filtrate fractions taken off will be determined by the number of fractions it is desired to make from the hydrolysate sample. We have made as many as 50 to 60 fractions in this manner, but experience has shown that, for our objectives, 10 to 20 fractions are all that it is worth while to make in the first fractionation. The fractions thus secured are precipitated by neutralization of the solvating acid reagent by addition of triethylamine, whereupon a certain portion of the dissolved amino acids is precipitated, as shown in Table II.

Fractionation with Amyl Acid Phosphate. When a sample of casein hydrolysate is fractionated with 0.2 N amyl acid phosphate in acetone according to the procedure described, the resulting fractions, when chromatographed, show that much simplification has occurred as compared with the original hydrolysate. The first increments of reagent which are added dissolve greater weights of hydrolysate per cubic millimeter than do later additions. These facts indicate that the solvating action of the reagent is a selective one, and that the undissolved fraction of the hydrolysate is continuously changing in composition as the fractionation proceeds.

Summary. Seven alkyl acid phosphates have been found able to solvate amino acids in dry acetone. Marked differences in solvating power are shown by these reagents for individual amino acids, and for selective solvation of amino acids from complex mixtures such as a protein hydrolysate. Mixtures of amino acids greatly influence each others' solubility when fractionation is effected by the method described.

When systems consisting of acetone-amino acid-solvating reagent, are neutralized with triethylamine, amino acids are precipitated, but never quantitatively. Marked differences occur in the extent to which the dissolved amino acids precipitate with this treatment; *e.g.* 90% were precipitated from the n-propyl, as against but 45% from the isopropyl system, when the amino acids were solvated from a dry casein hydrolysate.

These reagents offer prospects for usefulness in the fractionation of amino acid mixtures.

Received November 18, 1949. P.S.E.B.M., 1949, v72.

The Cardiac Output and Circulation Time of Ferrets.* (17552)

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The ferret was first used as a laboratory animal by Dunkin and Laidlaw for the study of canine distemper.(1) Subsequently it was also found to be susceptible, under experimental conditions, to other viral infections such as influenza,(2) fowlpox,(3) swineherd's disease,(4) Rift Valley fever,(5) a strain of poliomyelitis virus,(6) and lymphocytic chorio-meningitis.(7) In influenzal infection, experimental investigations employing ferrets have contributed a great deal to our knowledge of the pathology(8) and epidemiology(9) of this important disease. Other aspects of the infection, such as the problem of the toxicity of the virus and the alterations in the infected host's physiological functions are fields relatively unexplored. It seems that the ferret, because of its larger size, might serve better than the mouse or other rodents for investigations of these problems. Except for a general description by Pyle,(10) we are not aware of any detailed report dealing with the normal physiology of this animal. The present study dealing with the cardio-vascular function of normal ferrets was initiated in order to obtain such data before investigations of pathological physiology were undertaken. Cardiac output determined by the direct Fick principle, arterial blood pressure, and circulation time measurements were selected to depict the status of the animal's cardiovascular functions.

Materials and methods. 1. *Animals:* North American ferrets (*Putorius putorius*)‡ of both sexes with body weights varying from 700 to 1100 g were used. The body surface was calculated by the formula: (11) Surface area in square meters = (body weight in g)⅔ ×

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* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service.

† Research fellow in Medicine, University of Illinois College of Medicine.

‡ Identified by Mr. K. P. Schmidt, curator of zoology, Natural History Museum, Chicago.

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1/1000. The validity of the calculated value was carefully checked by actual measurements on the animals.

2. *Circulation time.* The experiments were performed in the morning on fasting animals. Anesthesia was induced by intraperitoneal injection of a solution of sodium nembutal, 30 mg per kg of body weight. Ten minutes later, the animal was placed on a board in supine position. Circulation time was then determined by the use of two technics: (a) A short incision was made in the left inguinal region and the femoral vein isolated. Five-tenths ml of a 10% sodium fluorescein solution was quickly injected into the vein. The interval between the beginning of the injection and the appearance of a green fluorescence in the oral mucous membrane, observed under ultra-violet light, was accurately timed; (b) A superficial vein in the neck was isolated and two-tenths ml of a 1% sodium cyanide solution was injected. The time from the beginning of the injection and the appearance of a deep inspiratory movement was noted.

3. *Cardiac output.* An incision was next made in the midline of the neck exposing the trachea for cannulation. The left common carotid artery was freed and cannulated with a No. 20 needle with a blunt bevel, connected to a mercury manometer which registered on a kymographic drum. The external jugular vein on the opposite side was dissected free and its cephalid end was tied. With the use of a specially constructed forceps(12) having sharp thin tips, an opening was made and held open in the wall of the vein. A No. 025 polythene catheter with metal stylet was introduced into the vein between the opened forceps. The catheter was advanced carefully toward the heart. The final position of the tip of the catheter was ascertained by placing the animal under a fluoroscope and with further manipulations the tip was made to lie in the right ventricle. The stylet was then removed and 0.2 ml of a 10% heparin solution in saline was introduced into the catheter to prevent clotting. The position of the catheter was fixed by tying a ligature around the vein.

The cardiac output was determined according to the direct Fick principle.§ The oxygen consumption was measured by connecting the tracheal cannula to a spirometer containing 100% oxygen. The volume of oxygen consumed was readily calculated from the kymographic tracing. As soon as the tracheal cannula was linked to the spirometer, a 1.5 ml sample of arterial blood was withdrawn from a three-way stopcock which was connected to the common carotid artery for the blood pressure determinations. A sample of venous blood (1.5 ml) was then quickly drawn from the polythene catheter. Both samples of blood were drawn into paraffined syringes and delivered immediately under a layer of paraffin oil in tubes. Determinations of the oxygen contents of these samples were made without delay according to the method of Van Slyke and Neil.(13) Five-tenths ml samples of blood were used for each determination and duplicates checked within 0.1 vol. %. Before the animal was killed, 1 ml of venous blood was taken from the polythene catheter, heparinized, and hematocrit readings were made by Wintrobe's method.

Results. Our findings (Table I) on the average rectal temperature and respiratory rate among the 10 normal animals were lower than those reported by Pyle(10) chiefly because our readings were made on anesthetized animals. Ferrets were easily excitable animals and such readings taken on unanesthetized animals were subject to wide fluctuations. The cardiac output, expressed in the form of cardiac index, was calculated in terms of liters per square meter of body surface per minute and was on the average 2.476 ± 0.75 liters. The average oxygen content of arterial blood was 15.7 vol. % and that of venous blood 10.2 vol. %. The average mean arterial blood pressure was 147

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12. Haas, E., and Goldblatt, H., *Science*, 1948, v108, 166.

§ Cardiac output =

$$\frac{\text{Oxygen consumption in ml per minute}}{(\text{Arterial O content}) - (\text{Venous O content})}$$

13. Peters and Van Slyke, *Quantitative Clinical Chemistry*, Vol. 2, p. 321.

TABLE I. Measurements of Circulatory Function of Normal Ferrets.

No.	Sex	Body wt, g	Rectal temp., °F	Respiratory rate, per min.	Pulse rate, per min.	O consumption, cc/min.	O content arterial blood vol., %	O content venous blood vol., %	A-V O difference, vol., %	Cardiac output, cc per min.	Body surface (m^2)	Cardiac index (liter/min./m^2)	Blood pressure, mm Hg	Fluorescein circulation time, sec.	Cyanide-circulation, sec.	Hematocrit, %
1	M	730	100	27	246	7.2	16.3	8.1	8.2	87.8	.052	1.68	120			
2	F	800	101.4	13	248	6.0	15.8	10.6	5.2	115.4	.055	2.09	156			
3	F	850	100.2	14		7.5	20.4	12.8	7.6	98.7	.057	1.75	138			
4	M	780	100.8	28	168	5.5	17.9	11.2	6.7	82.0	.054	1.51	134	9.0	3.9	49
5	M	1120	99.8	26	290	10.4	15.4	9.5	5.9	176.2	.067	2.61	185	8.1	3.1	35
6	M	800	99.3	21	168	3.8	18.7	15.5	3.2	118.7	.055	2.15	175	6.5	5.5	46
7	M	800	100.8	20	278	10.8	13.5	8.1	5.4	200.0	.055	3.62	133	4.5	4.9	41
8	F	800	98.0	32	250	7.9	13.2	8.1	5.1	154.9	.055	2.81	150	5.8	5.2	32
9	F	700	97.3	38	172	3.9	12.4	9.9	2.5	156.0	.051	3.06	150			41
10	M	850	97.0	58		9.6	13.2	8.3	4.9	195.9	.057	3.42	136			33
Mean		823	99.4	28.6	227	7.2	15.7	10.2	5.5	138.5		2.467	147	6.8	4.5	40
Stand. dev.		114	1.5	13.1	50.5	2.5	2.6	1.8	1.7			0.753	20	1.2	0.7	4.7

mm of mercury.

Circulation time measured by the fluorescein technic (6.8 sec.) was longer than that determined by the cyanide method (4.5 sec.), because of the greater distance the dye had to traverse when it was injected through the femoral instead of the superficial neck vein. The cyanide technic gave a sharper endpoint, although it has the objection of including the reaction of the respiratory center in a function test primarily for the circulatory system. The short circulatory time of ferrets rendered it very likely that technical errors such as the rate of injection and promptness in perceiving the endpoint would jeopardize the reliability of the results.

Discussion. In this group of animals, all the common conditions known to alter cardiac output as ingestion of food, hemorrhage, exercise and oxygen content of the inspired air(14) had been eliminated or made as uniform as possible. The only uncontrollable factor was the individual variations in response to the effect of the anesthetic used. In dogs, Shore(15) had shown that the effect of barbituric anesthesia on cardiac output was quite variable during the course of their experiments. The differences between successive determinations on the same animals were as high as 35%. Furthermore, anesthesia might cause hypoventilation or atelectasis, both of which would interfere with normal cardiac function. The individual variations in the cardiac output of our normal animals were partly technical and partly real. Our results agreed with those of Warren *et al.* on human subjects(16) in that arterial blood oxygen showed less variable results, provided that hematocrit readings were fairly constant. Greater degrees of variations, however, were encountered in the measurements of oxygen consumption. Because of these variations, they concluded that, "results are valid only when groups rather than individuals are studied."

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In a table compiled by Marshall(17) the cardiac indices for various animals were: Dog, 2.86; goat, 3.07; rabbit, 1.69; and horse, 5.84. The mean cardiac index of 2.476 for the present group of normal ferrets is one intermediate between those for dog and rabbit.

Summary. The mean cardiac output per minute per square meter of body surface for a group of 10 normal ferrets was found to be 2.476 liters. The average circulation time by the fluorescein technic was 6.8 seconds and 4.5 seconds as determined by the cyanide method. The average mean arterial blood pressure was 147 mm Hg. Various other related data were also tabulated.

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Received November 15, 1949. P.S.E.B.M., 1949, v72.

Amount of Alkaline Phosphatase in the Oviduct of Folic Acid Deficient Chicks.* (17553)

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Increases in alkaline phosphatase in the reproductive tract following injection of estrogens have been described for many species. Hertz and Sebrell(1) first noted an impairment in the response to stilbestrol of the oviduct of chicks deficient in folic acid. This failure of estrogens to induce growth in the absence of folic acid has also been observed in the monkey,(2) frog,(3) and rat.(4) The following experiments were undertaken to determine whether the amount of alkaline phosphatase in the oviduct of the chick is affected by a folic acid deficiency.

Material and methods. Exp. 1. Eleven white leghorn chicks were obtained at hatching and placed in cages having elevated wire mesh floors. At 2 days of age 5 chicks were placed on a diet of chick mash, while the remainder were fed chick mash containing 2% folic acid antagonist.‡ Both groups received water *ad libitum.* Three birds in each group were kept as uninjected controls. The remaining chicks were injected subcutaneously with 50μg of estradiol§ in 0.05 ml of sesame oil daily for 5 days. The injections were started on the 12th day of feeding and the chicks were killed 24 hours after the last injection. At post mortem the oviducts were removed, weighed and placed in cold 80% ethyl alcohol. Histochemical studies for alkaline phosphatase were carried out by the method of Gomori,(5) in which the sections were incubated at 37°C for 2 hours with glycerophosphate and examined without the use of a counterstain. Body weights were recorded daily and blood samples obtained at autopsy for hemoglobin determinations.

Exp. 2. A total of 38 white leghorn chicks were obtained at time of hatching and treatment started at 2 days of age. Thirteen of the chicks received the normal diet, 13 received the normal diet containing 2% folic acid antagonist and 12 received a synthetic

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* Aided in part by a grant from the American Cancer Society, Inc., to Harvard University.

† Present address: Department of Biological Sciences, Purdue University, Lafayette, Ind.

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‡ We are indebted to Dr. E. L. R. Stokstad, Lederle Laboratories, New York, for the crude x-methyl folic acid used in these studies.

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TABLE I.
Effect of Methyl Folic Acid on Deposition of Alkaline Phosphatase after Treatment with Estradiol.

No. of chicks	Diet	Estradiol, μg	Body wt, g	Oviduct wt, mg	Alkaline phosphatase
3	Control	—	95	17.3	0
2	”	50	112	29.0	3+
3	Antagonist	—	70	13.2	0
3	”	50	68	16.2	1+

TABLE II.
Effect of Crude Methyl Folic Acid and a Folic Acid Free Diet on Deposition of Alkaline Phosphatase after Treatment with Stilbestrol.

No. of chicks	Diet	Stilbestrol, mg	Body wt, g	Oviduct wt, mg	Alkaline phosphatase
6	Control	—	157	17.3	3.7+
7	”	0.5	159	436.3	3.7+
5	Synthetic	—	79	12.8	1.6+
7	”	0.5	71	78.5	1.7+
6	Antagonist	—	73	12.2	1.9+
7	”	0.5	68	69.2	0.7+

diet(6) supplemented with all the known vitamins except folic acid. Starting on the 13th day of feeding the special diets, 7 chicks in each of the 3 diet groups received 0.5 mg stilbestrol daily for 5 days. All chicks were killed 24 hours after the last injection and autopsied as in Exp. 1. In order to obtain information with regard to the relative amount of phosphatase present under the various conditions of the experiments an arbitrary scale was established ranging from 0 reaction for tissue totally devoid of phosphatase to a 4+ reaction for tissue showing a maximum response. In both experiments the same scale was used and the evaluation made only on the phosphatase noted in the endometrium.

Results. The chicks on the control diet showed a normal increase in body weight throughout the experiments whereas the birds on the synthetic diet and the diet containing the folic acid antagonist showed negligible increase in weight after the 9th day. Thus the final body weight of the chicks in Exp. 1 on antagonist diet was 69 g while the controls showed a body weight of 101 g. Similarly the control chicks in Experiment 2 reached a final weight of 158 g while the weight of the folic acid deficient chicks varied from 68 to 79 g (Tables I and II). Hemoglobin values varied widely; however, an average drop of 10% was obtained in the folic acid deficient birds. In Exp. 1, treatment with estradiol caused a 68% increase in the oviduct weight of the normal bird but had no effect on the weight of the oviduct of the chicks receiving the folic acid antagonist. Phosphatase occurred to a marked degree in the control chicks which showed a 3+ reaction and only to a slight degree, *i.e.*, a 1+ reaction, in the birds receiving the antagonist (Table I).

In Exp. 2 the higher dosage of estrogen used gave a much greater effect on the weight of the oviduct. The oviducts of the control chicks treated with stilbestrol weighed 436.3 mg whereas the oviducts of the chicks on the synthetic and antagonist diets weighed 78.5 and 69.2 mg respectively (Table II). The alkaline phosphatase was also markedly reduced in the oviducts of these last two groups. It is apparent from an examination of Fig. 1 that a large amount of alkaline phosphatase is present in the chicks on the normal diet whereas the folic acid deficient chicks show a marked reduction in the enzyme regardless of stilbestrol treatment.

Discussion. The present study confirms the work of Hertz(7) and others that folic acid is necessary for the growth-promoting

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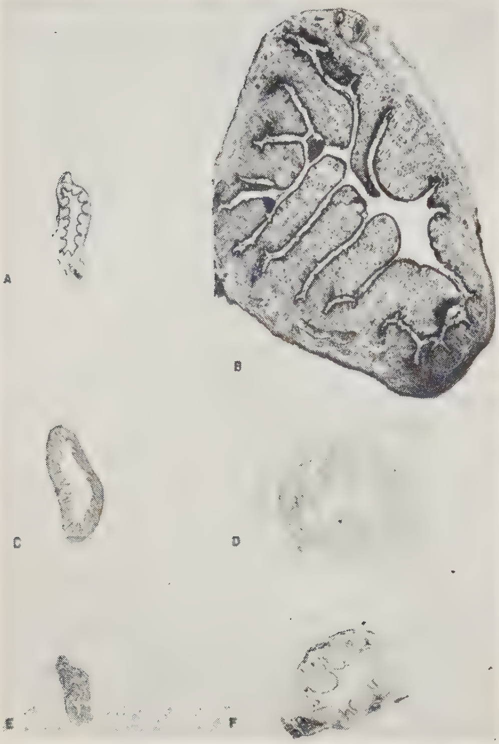

FIG. 1.

Sections through oviducts, all × 32. Note that the heavy deposition of alkaline phosphatase present in the endometrium of A and B, fails to occur in folic acid deficiency (C-F).

A—Uninj. control.
B—Normal diet. Inj. with stilbestrol.
C—Synthetic diet.
D—Synthetic diet. Inj. with stilbestrol.
E—Antagonist diet.
F—Antagonist diet. Inj. with stilbestrol.

action of estrogen on the reproductive tract. The mechanism whereby this vitamin acts in the growth of the tissue is as yet unexplained.

It has been suggested that folic acid may function as a coenzyme in the synthesis of thymine-like compounds which are then utilized in the production of nucleic acid.(8) Partial support for this concept is seen in the work of Prusoff *et al.*(9) who showed that a decrease in desoxyribonucleic acid occurred when *Lactobacillus casei* is grown on a folic acid deficient medium. It has also been shown that aminopterin not only inhibited the estrogen induced growth of the oviduct of the frog but caused an increase in the number of mitotic figures, conceivably by a reduction in nucleic acid and consequent retardation in cell division.(10) The present results indicate that folic acid may also be essential for the deposition and maintenance of alkaline phosphatase in the reproductive tract. If this enzyme is necessary for the growth-promoting action of estrogen then its absence would explain the failure of estrogens to induce growth. Thus it is possible that the absence of folic acid as induced under the conditions of this experiment interferes with the increase in phosphatase following estrogen treatment and prevents normal growth response.

Summary. A folic acid deficiency produced by feeding a synthetic diet or the addition of crude x-methyl folic acid to a normal diet markedly decreases the amount of alkaline phosphatase in the oviduct of chicks after estrogen treatment.

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Received November 1, 1949. P.S.E.B.M., 1949, v72.

Effects of X-Rays and Nitrogen Mustard on Lymphatic Nodule of the Rabbit.* (17554)

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The relationships between the dose and the histologic effects of total body radiation with X-rays on the lymphatic nodule has been reported previously.(1) It was found that the sequence of histologic changes in the lymphatic nodules is characteristic for different dose levels. Total body radiation of rabbits with 800r and 600r at a rate of 16r per minute produces a rapid and complete destruction of the lymphatic nodule resulting in a nodule-free period beginning at 5 days with a subsequent regeneration at 21 days after radiation. Lower doses (400r and lower) produce only a partial destruction of the lymphatic nodule with an immediate regeneration so that no nodule-free period occurs. For a detailed description of the changes in the lymphatic nodule after X-rays, we refer to previous papers.(1) The similarity between the hematotoxic effect of X-rays with that of nitrogen mustard is well known and has been reported by a number of investigators. It seemed to us of interest to investigate whether this similarity extends itself to the histologic effects on the lymphatic nodule and if so, to compare, on this basis, the degree of the effect on the lymphopoietic system of these two agents with respect to their general toxicity to the organism. The nitrogen mustard compound used for this investigation is methyl bis (B-chloroethyl) amine hydrochloride.

The LD_{50} dose is taken as a measure of the general toxicity of X-rays and nitrogen mustard. These are respectively 800r ($LD_{50}/30$ days)(2) and 2 mg per kg ($LD_{50}/14$ days).(3) The 0.1% solution of nitrogen mustard was injected immediately after the crystals were completely dissolved in view of the instability of the compound. Eighteen rabbits were injected intravenously with 2 mg per kg nitrogen mustard. Eleven rabbits were killed at the following intervals: 30 min.; 3 hr; 8 hr; 16 hr; 24 hr; 3 days, 5 days, 10 days, 21 days, 6 weeks and 2 months. After the first 4 animals were killed, 7 of the remaining 14 died within 14 days after the injection. This indicates that the given dose was in the range of the LD_{50} for this group of animals. The lymphatic nodules of the mesenteric lymph node and the appendix were studied.

The sequence of events in the lymphatic nodule of the mesenteric lymph node of the rabbit after nitrogen mustard is essentially identical with that after irradiation with the LD_{50} dose. The destruction of lymphocytes leads to a nodule-free period beginning at 5 days and which terminates at 21 days when regenerative processes are present as evidenced by the formation of new nodules. Comparing the degree of regeneration with that after 800r of X-rays at the same interval, it appears that the regeneration is more advanced after nitrogen mustard. At 21 days there are larger and more new lymphatic nodules. These lymphatic nodules are at this time still without corona of small lymphocytes ("bare" germinal centers). A corona is present at 6 weeks and the regeneration is complete from then on.

In contrast to the changes in the mesenteric lymph node, the changes in the appendix after the LD_{50} dose of nitrogen mustard are not comparable with those after an LD_{50} dose of X-rays but they are similar to the results after the lower dose levels (400r and less). There is no nodule-free period, but after the debris has cleared up, small groups of lymphocytes remain in the dense masses of reticular

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* This work was supported by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

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3. Unpublished data, Univ. of Chicago Toxicity Laboratory.

cells. Regeneration occurs from these remaining lymphocytes.

These observations indicate that the histologic effect of nitrogen mustard on the lymphopoietic system is qualitatively but not quantitatively the same as the effect of X-rays at the same level of general toxicity. The effects on the mesenteric lymph node differ only slightly, the difference being a somewhat earlier regeneration. The difference is greater in the lymphatic tissue of the appendix. This lymphatic tissue shows changes after nitrogen mustard at the LD_{50} comparable to the changes of the X-rays with a dose of half the LD_{50}. The findings of Jacobson *et al.*(4) demonstrating the more rapid regeneration of the rabbit bone marrow after nitrogen mustard as compared to X-rays and the findings reported here, seem to warrant the conclusion that the hematopoietic system regenerates in general more rapidly after nitrogen mustard than after X-rays with dose levels of the same general toxicity, keeping in mind local differences such as are reported here for the mesenteric lymph node and the lymphaic tissue of the appendix. We have no definite explanation for these local differences. They may be based on differences in sensitivities of the lymphocytes, but it may also be that the instability of the compound *in vivo* causes differences in the effective concentrations in different organs.

Summary. The histologic effects on the lymphatic nodules of the rabbit after X-rays and after nitrogen mustard are identical in nature. However, there is a quantitative difference in the response of the lymphatic nodules to the LD_{50} dose of the two agents. The difference is minor in the mesenteric lymph node, where there is a slightly earlier regeneration after nitrogen mustard than after X-rays. The difference is greater in the appendix where the histologic changes after nitrogen mustard at the LD_{50} dose level are comparable to the changes after half the LD_{50} dose of X-rays.

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Received November 4, 1949. P.S.E.B.M., 1949, v72.

Effect of Cortisone on Production of Granulation Tissue in the Rabbit.*
(17555)

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Since Hench and his co-workers(1) demonstrated the dramatic therapeutic effect of cortisone and adrenocorticotrophic hormone (ACTH) in rheumatoid arthritis, numerous investigators have found that these agents exert beneficial effects in a great array of disorders. These include such "mesenchymal diseases" as rheumatic fever, lupus erythematosus disseminatus and periarteritis nodosa. (2-4). The mechanism by which therapeutic

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* Supported in part by the Masonic Foundation for Medical Research and Human Welfare, the U. S. P. H. S., and a grant from the Medical Research and Development Board, Office of the Surgeon General, U. S. Army.

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TABLE I.
Blood Chemical Determinations on 4 Cortisone-treated Rabbits and 4 Control Rabbits Following Production of Ear Wounds.

	5th post-operative day				8th post-operative day			
	Cortisone-treated rabbits		Control rabbits		Cortisone-treated rabbits		Control rabbits	
	No. 55	No. 57	No. 52	No. 56	No. 54	No. 58	No. 53	No. 59
Serum protein (g%)	5.1	5.0	—	—	5.7	5.1	5.5	6.0
Serum albumin (g%)	4.2	4.3	—	—	3.8	4.1	3.8	4.1
Serum sugar (mg%)	235	170	235	122	294	129 (fasting)	174	122 (fasting)
Plasma vit. C (mg%)	—	.42	—	.65	—	0.34 (fasting)	—	.02 (fasting)

effects are achieved has not, up to the present, been clarified, but it has appeared to the writers that the action of these agents is on "host reactivity."(5) In the course of treatment of several patients with lupus erythematosus disseminatus and rheumatoid arthritis with ACTH, it was noted that open wounds, such as incised abscesses and decubitus ulcers, failed to show normal granulation tissue.(5) One such wound, an incised abscess, did not form granulation tissue until 4 days after ACTH was discontinued. Healing of aseptic wounds, made for biopsy, was greatly delayed while ACTH was administered, but these healed promptly when the hormone was discontinued. These observations raised the question of whether the action of ACTH and cortisone in the "mesenchymal diseases" might be due to "inhibition of reactivity" of the connective tissue. Patients with hyperadrenalism as manifested by Cushing's syndrome are known to have a disturbance of connective tissues as exemplified by the "moon" facies, formation of striae, osteoporosis, increased fragility of blood vessels and poor healing of wounds. To test this hypothesis it was decided to determine whether the administration of cortisone might inhibit the normal processes of granulation in artificially produced wounds.

Skin wounds were made on both ears of six pairs of rabbits weighing between 2.4 kg and 3.0 kg.(6) One rabbit of each pair was treated with cortisone according to the following schedule: 12.5 mg of cortisone acetate was administered intramuscularly twice daily for 3 days before the wound was made and for 5 to 8 days thereafter until the time of sacrifice. The other rabbit of each pair served as a control and received no cortisone but was otherwise treated exactly the same. The animals were kept in a thermostatically controlled room and identical local treatment to prevent wound infection, as reported elsewhere,(6) was carried out in each pair. Control and cortisone-treated rabbits ate a similar diet, both groups ate well and weight remained relatively constant.

The development of granulation tissue in the cortisone-treated animals was markedly delayed in all cases. Grossly, at both 5 and 8 days following operation, these rabbits showed little or no granulation tissue and the blood vessels of the wound stood out clearly, while the controls showed good granulation tissue which obscured the blood vessels. Two pairs of rabbits were sacrificed on the fifth post-operative day and two pairs were sacrificed on the eighth post-operative day. The remaining two pairs were not sacrificed but showed similar gross changes and are being used for further long-term experiments. The cortisone-treated rabbits developed such manifestations of hyperadrenalism as the disappearance of circulating eosinophils, a moderate post-prandial hyperglycemia and a great increase

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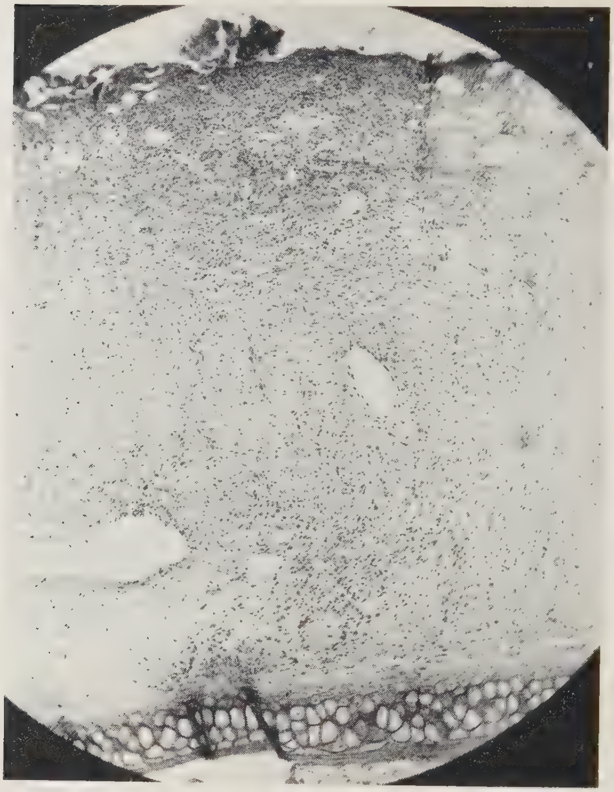

FIG. 1.
Cross-section of rabbit's ear wound, 110 ×, eighth post-operative day, control rabbit.
At the base is the cartilage of the rabbit's ear. Note the great thickness of the granulation tissue, the presence of newly-formed blood vessels and the elaboration of reticulin fibrils.

in glycogen deposition in the liver. Serum protein partitions in the animals receiving cortisone were comparable to control animals. (Table I). There was no evidence of wound infection in either the cortisone-treated or control rabbits.

Histologically, the cortisone-treated rabbits at both 5 and 8 days post-operatively showed striking depression of new growth of all elements of the connective tissue. (Fig. 1 and 2). The height of the granulation tissue formed was markedly decreased. Few, if any, new blood vessels could be seen. On the eighth post-operative day, fibroblasts in compact arrangement were present in nests about old blood vessels. In the control animals the new fibroblasts were present throughout the wound and appeared to be much less compact. Fibrils appeared between the fibroblasts and there was more ground substance as determined roughly by metachromasia with toluidine blue stains and by the Hale(7) stain.

This suppression of all new elements of the connective tissue is similar to that seen in scurvy. Plasma ascorbic acid levels in the cortisone-treated rabbits tended to be somewhat lower than the control rabbits but the levels did not reach zero in the plasma. In this connection it is of interest that low ascorbic acid levels have been observed in our clinic in three patients with Cushing's syndrome. In the production of scurvy by deficiency of vitamin C, complete disappearance of this vitamin from the plasma is found

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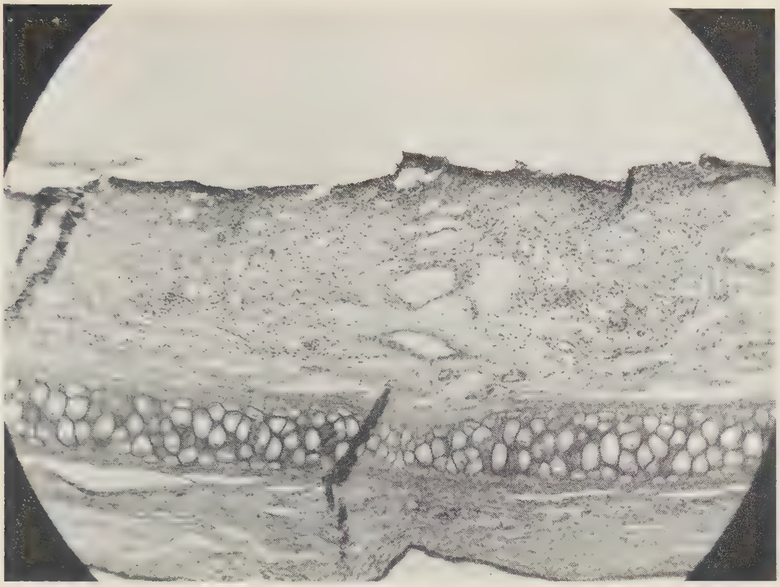

FIG. 2.

Cross-section of rabbit's ear wound, 124 ×, eighth post-operative day, cortisone-treated rabbit.

Pre-formed blood vessels left in the connective tissue at the time the skin was removed may be seen in the center of the field. Note the narrow area of granulation tissue with densely-packed fibroblasts.

months before any interference with wound healing is noted. Thus if this effect be related to vitamin C deficiency the result might be due to impairment of utilization of available ascorbic acid. No manifestations of the hemorrhagic diathesis were observed in the animals receiving cortisone. This correlates well with clinical findings in humans since no manifestations of scurvy have developed in patients we have treated with ACTH for as long as 4 months, and 600-900 mg of ascorbic acid administered daily to patients being treated for rheumatoid arthritis with ACTH or cortisone have failed to change the beneficial effects. However, it is of interest that preliminary experiments in the rat, an animal which is resistant to the development of vitamin C deficiency, show that wound healing is not modified by the administration of cortisone in a dose of 1 mg per day per 100 g of rat. It is noteworthy also that the rat is remarkably resistant to histamine while the rabbit is a histamine-sensitive animal. Rats in general are refractory to the pharmacological action of most drugs and it is possible that the dose of cortisone was too low to affect wound healing. Further studies are in progress to clarify this species difference as well as to attempt to "titrate" the minimal dosage of cortisone necessary to inhibit the growth of granulation tissue in the rabbit.

Summary. 11 dehydro 17 hydroxycorticosterone (Compound E of Kendall, cortisone) administered to rabbits in large doses caused a delay in the development of all elements of connective tissue. It is suggested that the action of cortisone and ACTH on the "mesenchymal diseases" is due, at least in part, to "inhibition of reactivity" of the connective tissue. It is furthermore suggested that the inhibition of the growth of granulation tissue in the rabbit may prove of value in assay of compounds active in the treatment of "mesenchymal diseases."

Received November 18, 1949. P.S.E.B.M., 1949, v72.

The Development of Hypertension and Nephritis in Normal and Adrenalectomized Rats Treated With Cortisone.* (17556)

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The following studies were undertaken to determine the influence of cortisone upon the development of hypertension and nephritis in rats. Observations have been made upon adrenalectomized as well as intact animals.

Procedure. Twenty-three 2 to 3 months old male and female rats of the Long-Evans strain were divided into 3 groups. In the 8 animals in each of the first 2 groups the adrenals were intact, while the seven animals of the third group were bilaterally adrenalectomized at the beginning of the experimental period. The animals were fed a modification of the McCollum stock diet,(1) adjusted to contain 1.7% sodium chloride. In addition, all 3 groups received 0.85% saline as drinking water. All animals were injected intravenously on 3 successive days with rabbit anti-rat kidney serum,(2) totalling 0.8 cc. Group I received only the cytotoxic serum; Group II received, in addition, daily subcutaneous injections of 2.5 mg of cortisone starting one week prior to the administration of anti-kidney serum. In Group III, cortisone was begun 8 days after adrenalectomy and immediately following the anti-kidney serum injections, in the dosage of 5 mg daily; after the first week, the daily dose was reduced to 2.5 mg. Blood pressure determinations were made at approximately weekly intervals on all rats. The indirect plethysmographic method originally described by Williams, Harrison and Grollman(3) and later modified by Sobin(4) was employed and the results are recorded as previously outlined by these authors.(5) The growth of the animals was recorded. At the conclusion of the period of observation, 4 to 5 weeks after the administration of anti-kidney serum, the rats were anesthetized with ether, without prior fasting, then bled and sacrificed. Determinations of serum sodium were made using a flame photometer with an internal standard having an accuracy of ±1%.(6) In addition, determinations of urea nitrogen were made in all groups, and, in the 2 groups of intact rats, of serum carbon dioxide content, chlorides, sugar and total cholesterol using standard methods. The weights of heart, kidneys, thymus and the adrenal glands were recorded. No adrenal tissue was found in the operated animals (Group III). Sections of kidney were fixed in Zenker's fluid and stained with hematoxylin and eosin for histological study. Sections of the pituitary were stained by Masson's technic.

Results. In Fig. 1 growth curves are presented. In the adrenalectomized group a most

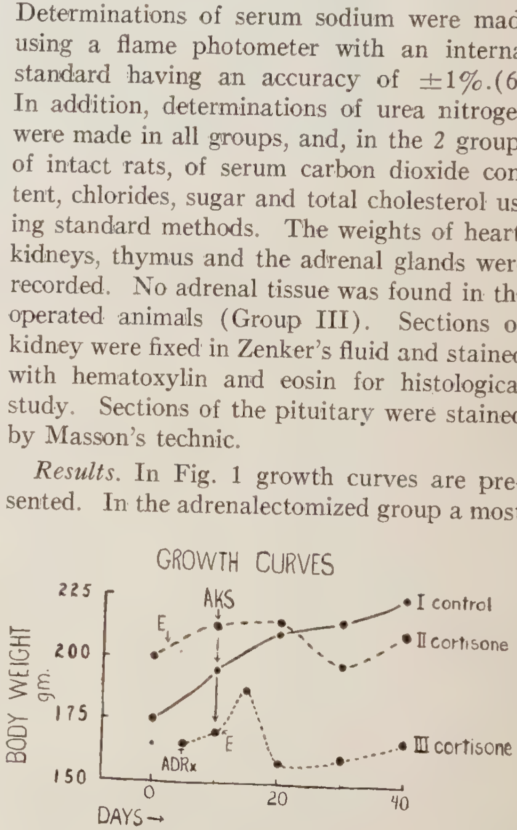

Fig. 1.
AKS = Rabbit anti-rat kidney serum.
ADR$_x$ = Bilateral adrenalectomy.
E = Date on which cortisone injections were commenced.

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* This investigation was supported, in part, by a research grant from the National Heart Institute, U. S. P. H. S., and was aided through the generosity of the Albert and Mary Lasker Foundation.

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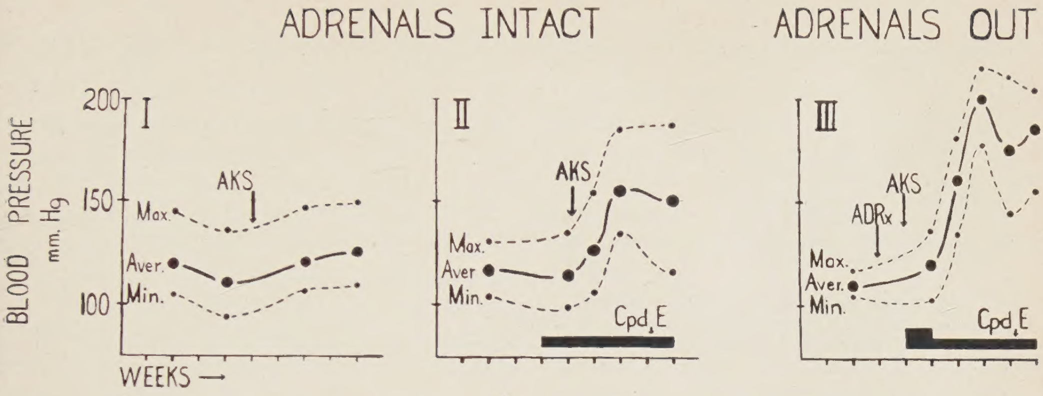

FIG. 2.

Effect of cortisone on the blood pressure of intact and adrenalectomized rats given anti-kidney serum.

Aver. = Avg weekly blood pressure of all rats in group.

Max. and Min. = Highest and lowest individual blood pressure readings each week.

AKS = Rabbit anti-rat kidney serum given at time indicated by arrow.

ADR$_x$ = Bilateral adrenalectomy performed at time indicated by arrow.

Cpd E = Cortisone. Duration of administration indicated by solid black bar.

impressive weight gain was evident within the first week after serum administration while the rats were receiving 5 mg of cortisone daily. This correlated with the development of edema in these animals. Subsequently and concomitantly with a decrease in cortisone dosage to 2.5 mg daily, the edema disappeared with a corresponding loss of weight. The differences in growth of Groups I and II are of doubtful significance, owing to the fact that the rats of Group II had attained greater growth than those of Group I when the experiment was begun.

In Fig. II the blood pressure readings are presented in graphic form. No significant change in blood pressure occurred during the 4 weeks of observation after the administration of anti-kidney serum in the group which did not receive cortisone (Group I). In the second group, which received cortisone, a moderate rise in blood pressure occurred during a similar period of time. These changes were in the same direction but less striking than had been observed previously in nephritic rats given DCA in comparable dosage.(5) In the group of adrenalectomized rats given cortisone (Group III), hypertension appeared and was of a much more impressive degree than in Group II.

In Table I the results of the chemical determinations are tabulated. Comparison of the values obtained in the first and second groups shows no significant differences with the possible exception of the serum cholesterol, which was somewhat more elevated in the group receiving cortisone. In the adrenalectomized group (III) given cortisone, the highest value for serum sodium was obtained.

The averages and range of weights for the various organs are presented in Table II. Because of the varying size of the animals and because the groups were comprised of varying numbers of males and females, interpretation of the results is difficult. However, cardiac and renal hypertrophy was not observed in the cortisone treated groups (II and III) in the degree previously noted with DCA.(5) On the other hand, the well known atrophy of thymus and of adrenals was apparent in the cortisone injected animals.

In Table II the data pertaining to histological examination of the kidneys is included. The majority of animals in all 3 groups showed evidence of nephritis, involving glomerular, tubular and interstitial elements. Thus, cortisone failed to prevent the establishment of nephritis caused by anti-kidney serum. A comparison of the severity of the nephritis in the different groups is hazardous because of the small number of animals employed and because this form of renal damage exhibits considerable variation from animal to animal.

TABLE I.
Results of Chemical Determinations.

Group	I	II	II
Adrenals	Intact	Intact	Out
Regimen	AKS	Cortisone and AKS	AKS and Cortisone
Serum sodium, mEq/L	136.4*	139.2*	143.5†
Serum CO_2 content, mEq/L	23.0*	24.8*	—
Serum chlorides, mEq/L	98.0*	98.2*	—
Serum sugar, mg/100 cc	174.0*	164.0*	—
Serum urea nitrogen, mg/100 cc	22.0*	24*	22†
Serum cholesterol, mg/100 cc	282*	330*	—

AKS = Rabbit anti-rat kidney serum.
* Results based on pooled sera from all animals.
† Results based on average of individual determinations on 6 animals.

TABLE II.
Body and Organ Weights, Incidence of Nephritis.

Group	I	II	III
Adrenals	Intact	Intact	Out
Regimen	AKS	Cortisone and AKS	AKS and Cortisone
No. of rats in group	8	8	7
No. of ♂/♀	4/4	5/3	5/2
Body wt at sacrifice (g)			
Avg	225	210	162
Range	170-346	152-265	145-179
Kidney wt (g)			
Avg	2.71	2.76	2.46
Range	1.95-3.36	1.94-3.68	1.50-4.24
No. of animals showing nephritis	7/7†	5/6†	5/7
Heart wt (g)			
Avg	.89	.91	.82
Range	.77-.98	.75-1.13	.66-1.18
Thymus wt (mg)			
Avg	141	46	49*
Range	105-173	21-87	38-61
Adrenals wt (mg)			
Avg	52.8	31.1	
Range	42.8-67.6	18-44.4	

AKS = Rabbit anti-rat kidney serum.
* Avg of 6 animals.
† Tissues of remaining animals lost.

The lesions appeared to be of no greater severity among the two hypertensive groups (II and III) than among the normotensive group.

Dr. Philip E. Smith, who was kind enough to examine the pituitary glands of these rats for evidence of hyalinization of basophilic cells as described by Crooke in Cushings Syndrome, reported no significant differences between control and cortisone injected animals. However, he has emphasized that the histological differentiation of rat pituitary gland is not wholly satisfactory.

Discussion. Cortisone has been reported to cause striking remissions in certain diseases in which antigen-antibody reactions are believed to play a role. The present study was designed to investigate whether the injection of this steroid might interfere with the development of a cytotoxic serum nephritis, the mechanism for which is likewise presumed to depend upon some form of sensitization. The results indicate that the administration of cortisone, even when given prior to the injection of antikidney serum, as in Group II, failed to prevent the development of nephritis. Since this form of nephritis represents, at least initially, the result of the introduction of preformed antibodies, these findings do not exclude the possibility that cortisone may influence the mechanisms involved in active antibody production.

In experiments with nephritic rats previously reported,(5) desoxycorticosterone acetate (DCA), when injected daily in doses of 2.5 mg over a period of weeks, produced striking hypertension. The present study indicates that cortisone, a steroid which differs from DCA in many of its physiological properties, has an effect upon the blood pressure qualitatively similar to DCA. However, in the animals with intact adrenal glands, the hypertension produced by cortisone was far less impressive than that seen previously with DCA, particularly so since in the present study a considerably higher sodium chloride intake was given. On the other hand, in the adrenalectomized rat given cortisone the hypertension is striking, and suggests the possibility that in the intact animals, the presence of other adrenal steroids tends to counteract the pressor effect of this steroid. The development of hypertension during cortisone administration in the adrenalectomized rat with renal damage is in keeping with the previous work of Guadino.(7) Working with rats made hypertensive by a combination of unilateral nephrectomy and wrapping the remaining kidney with gauze and collodion, he observed a fall in blood pressure to follow adrenalectomy. Subsequently, cortisone injections led to a reappearance of the hypertension.

In the rats currently reported, the striking hypertension which appeared in the cortisone treated adrenalectomized group could not be correlated with a greater degree of nephritis in these animals inasmuch as renal lesions of comparable severity were observed in the other two groups.

Summary. 1. The administration of cortisone did not prevent the development of experimental cytotoxic serum nephritis in the rat.

2. Moderate hypertension developed in nephritic rats with intact adrenal glands when injected with cortisone, whereas striking hypertension appeared in adrenalectomized nephritic rats similarly treated.

3. The nephritic rats rendered hypertensive by the administration of cortisone presented no greater histological evidence of renal damage than did normotensive control nephritic animals.

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Received October 31, 1949. P.S.E.B.M., 1949, v72.